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# STUDIES ON BIOLUMINESCENCE.

## XV. Electroreduction of Oxyluciferin.

By E. NEWTON HARVEY.

*(From the Physiological Laboratory, Princeton University, Princeton.)*

(Received for publication, August 28, 1922.)

In previous papers I have pointed out (1918, 1920) that the light-producing reaction in the luminous crustacean, *Cypridina*, is probably to be represented by some such equation as

$$\text{Luciferin} + \text{oxygen} = \text{oxyluciferin} + \text{water}$$

or, symbolically

$$LH_2 + O = L + H_2O$$

Luciferin is autooxidizable and the equation proceeds in the right-hand direction spontaneously in presence of oxygen. No luminescence appears, however, unless luciferase, a thermolabile product of *Cypridina*, is also present. Whether luciferase is present or absent, the same oxidation product, oxyluciferin, is formed. Luciferase undoubtedly acts as a catalyst and accelerates the reaction, but in addition, is absolutely necessary for the production of light. A succeeding paper deals with the oxidation of luciferin.

Oxyluciferin can be reduced under the influence of reducing agents when hydrogenation can occur. Symbolically

$$L + H_2 = LH_2$$

The luciferin is thus regenerated. I found that reduction may be brought about by hydrogen sulfide or other sulfides, by the action of acids or alkalies on metals, whereby nascent hydrogen is produced, by palladium and sodium hypophosphite, and by reducing enzymes. The usual procedure was to add the reducing agents to a solution of oxyluciferin made by thoroughly boiling an extract of *Cypridina* in order to destroy the luciferase and oxidize all the

luciferin to oxyluciferin. After allowing the oxyluciferin solution to stand for some time in contact with a reducing agent, the presence of luciferin could be detected by adding luciferase solution and noting if any light occurred. If anyone doubts that reduction of the oxyluciferin is really the phenomenon observed in these experiments, I think the following observations are sufficient to prove conclusively the reality of reduction.

It is a well known fact that reduction occurs at the cathode and oxidation at the anode when an electric current is sent through a solution. If, between platinum electrodes, a current is sent through an extract of *Cypridina* containing luciferase and oxyluciferin and some NaCl, to make it a good conductor, a beautiful luminescence appears over the surface of the cathode, and none over the anode. With N NaCl, the luminescence is just visible at 1.8 volts across the electrodes and is marked at 2 volts. This is about the potential at which hydrogen first appears in a N NaCl solution and is very slowly liberated as bubbles from the electrode. The potential difference necessary for luminescence varies with the nature of the metal constituting the cathode. With a platinized platinum cathode and smooth platinum anode, the potential difference to give a just visible luminescence was 2 volts.

The explanation of this experiment is as follows: Nascent hydrogen is liberated through decomposition of water by the Na set free at the cathode.

$$Na + HOH = NaOH + H$$

The nascent hydrogen, H, reduces the oxyluciferin to luciferin, which then oxidizes with luminescence in contact with luciferase and oxygen, also present in the solution. Thus we have a layer of luciferin formed in immediate contact with the electrode and just beyond this the oxidation of the luciferin to oxyluciferin with luminescence. The NaOH formed is too small in amount to inhibit the action of the luciferase. If the NaOH formation is too rapid the luminescence disappears, since the layer of luciferase in contact with the electrode is destroyed or inhibited by the alkali. This occurs when the potential difference across the electrodes is increased. By the use of an oxyluciferin solution containing luciferase and oxygen we have a

means of immediately determining that reduction of the oxyluciferin has occurred, which is more convenient and rapid than former methods, in which I first reduced an oxyluciferin solution in the absence of oxygen and then mixed it with luciferase solution containing oxygen. The present method is especially applicable to the study of reductions at the surfaces of solids and gives us an interesting insight, as will be seen later, into the action of heterogeneous catalysts

Not only may the luminescence under the above conditions serve as an indicator of reduction of oxyluciferin, but conversely the luminescence may serve as an indicator of nascent or atomic hydrogen. Hydrogen from a cylinder will give no luminescence whatever if bubbled in a fine stream or shaken (so as to break it up into very minute bubbles) with oxyluciferin-luciferase-oxygen solution. Only if the hydrogen is liberated in the oxyluciferin-luciferase-oxygen solution so that the hydrogen possesses its "active" qualities, will luminescence occur. The "active" qualities persist only within very small distances from the surface of separation, as can be easily demonstrated by this luminescent reaction. The "active" hydrogen is no doubt atomic hydrogen which changes rapidly in the sense of the equation

$$2\,H = H_2$$

If oxyluciferin can be reduced at cathodes during electrolysis it should be possible to observe reduction of oxyluciferin when present in a fluid serving as the electrolyte of a voltaic cell, in which two dissimilar metals are placed. It should also be possible to observe reduction of oxyluciferin at the surface of metals capable of liberating hydrogen from water. Both possibilities can be realized.

The following metals, arranged in the electrochemical series, were available and have been tried: Na, Ca, Mg, Al, Cr, Mn, Zn, Cd, Fe, Co, Ni, Sn, Pb, (H), Sb, Bi, As, Cu, Hg, Ag, Pd, Pt, Au, C. It is well known (1) that this series has the greatest action in decomposing water in the order indicated, (2) that the metal standing foremost in the series will precipitate the metals following it in the series from solutions of their salts, and (3) will give rise to an electric current with any other metal of the series, when dipped into an electrolyte and the metals connected. The current is of such a nature

that the foremost metal will be negative and the succeeding metal positive, the foremost metal forming the anode, and the succeeding metal the cathode of the voltaic cell.

In line with these statements I find that Mg, Al, Mn, Zn, and Cd, placed in oxyluciferin-luciferase-oxygen solution, luminesce over their fresh cut surfaces. None of these metals liberates visible hydrogen from water at room temperature except Mg, but a thin film of atomic H must be formed over their surface which is replaced as used in reducing oxyluciferin, for the luminescence may last for some time.

Mg gives off visible $H_2$ and dissolves to form $Mg(OH)_2$ with quite an alkaline reaction. This alkalinity slowly destroys the luciferase so that the luminescence ceases after a time. Na[1] and Ca liberate hydrogen violently with such rapid formation of strongly alkaline NaOH and $Ca(OH)_2$, that the luciferase is rapidly destroyed and no luminescence is possible.

The Zn and Al used were prepared by the Bureau of Standards at Washington and were the purest that can be obtained.[2] They gave a rather brighter luminescence than other samples of less pure metals so that the luminescence is probably not connected with "local action" currents over the surface of the metal.

As I have previously indicated, all reduction processes must be carried out in approximately neutral media if we are to test the production of luciferin from oxyluciferin by means of luciferase, itself so sensitive to acid or alkali. For this purpose the solution of Mg in ammonium salts is a convenient method. Mg dissolves in ammonium salts with the rapid evolution of nascent H and formation of a double salt of practically neutral reaction. This forms a convenient and efficient method of reducing oxyluciferin. The Mg wire glows beautifully, while bubbles of hydrogen pass upward through the oxyluciferin-luciferase solution. After a time all the oxygen is displaced and the luminescence then ceases. A large amount of luciferin now accumulates, since its oxidation is prevented, and this can be oxidized with quite a brilliant luminescence by merely inverting or shaking the tube so as to dissolve more oxygen.

[1] Na luminesces of its own accord on dissolving in water but this luminescence has nothing to do with reduction of oxyluciferin.

[2] I am indebted to Professor Donald Smith of the Chemistry Department, Princeton University, for samples of these metals.

Another convenient method is the use of aluminum-amalgam.[3] Aluminum-amalgam dissolves in water with the continuous formation of nascent hydrogen and insoluble Al $(OH)_3$ or $Al_2O_3$. If oxyluciferin is also present it is reduced to luciferin which gives a bright light with luciferase.

It would seem that these two methods of reduction in neutral solution might find a more widespread use in biochemistry where one wishes to avoid the often destructive effects of H and OH ions.

We are now in a position to describe the luminescent effects at galvanic couples formed in oxyluciferin-luciferase-oxygen solutions plus N NaCl, to render the solution a conductor. If a strip of Zn or Al or Cd is placed in such a solution, the metals glow spontaneously as before described. A piece of platinum placed in the solution does not glow, but if the Zn, Al, or Cd is touched to the platinum, the platinum luminesces quite brightly, with greater intensity than the Zn, Al, or Cd alone. At the same time the luminescence of the Zn, Al, or Cd either fades or disappears. The platinum forms the cathode of the galvanic circuit at which reduction processes occur, the oxyluciferin is reduced to luciferin in immediate contact with the platinum and then is oxidized again with luminescence by the luciferase and oxygen layer next to it.

I have tried out a series of metal combinations in oxyluciferin-luciferase-NaCl-oxygen solution with the results tabulated in Table I. It will be noted that there is no relation to the calculated E.M.F. which such cells should give, based on electrode potentials in N solution of the ions of the metal in question. Thus, Zn touched to Fe will cause the Fe to glow when the E.M.F. is only $0.77 - 0.46 = 0.31$ volt, while Fe touched to Pt will not cause the Pt to glow although the E.M.F. is $0.46 + 0.86 = 1.32$ volts. Only those metals which luminesce spontaneously in oxyluciferin-luciferase solution will cause a nobler metal in contact with them to luminesce.

Attention should also be called to the fact, that, judging by its position in the electrochemical series, Cr should luminesce spontaneously, but does not show any indication of luminescence in contact with oxyluciferin-luciferase solution. This unexpected behavior

[3] To prepare this it is sufficient to dip aluminum shavings or wire for a few minutes in 0.5 per cent $HgCl_2$.

TABLE I.

*Luminescence Obtained When Metals and Metal Couples Are Placed in Oxyluciferin-Luciferase Solution.*

| Metal. | Normal potential. | Spontaneous luminescence. | Luminescence obtained when metal of first vertical column is touched with | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Zn | Cd | Fe | Cu | Ag | Au |
| Mg | −1.48 | + | Both. | Cd brighter. | Fe | Cu | Ag | Au |
| Al | −1.28 | + faint. | " | Cd " | Fe faint. | Cu faint. | Ag faint. | Au faint. |
| Cr | | − | Zn | Cd | − | − | − | − |
| Mn | −1.07 | + | Both. | Cd brighter. | Fe | Cu | Ag | Au |
| Zn | −0.77 | + | | Cd " | Fe | Cu | Ag | Au |
| Cd | −0.42 | + faint. | Both. | | − | Cu faint. | Ag faint. | Au faint. |
| Fe | −0.46 | − | Fe | − | | − | − | − |
| Co | −0.30 | − | Co | − | − | − | − | − |
| Ni | −0.25 | − | Ni | Ni faint (?). | − | − | − | − |
| Sn | −0.19 | − | Sn | Sn " (?). | − | − | − | − |
| Pb | −0.12 | − | Pb | Pb " | − | − | − | − |
| H | ±0.00 | − | | | | | | |
| Sb | | − | Sb faint. | Sb faint. | − | − | − | − |
| Bi | | − | Bi " | Bi " | − | − | − | − |
| As | | − | As " | As " | − | − | − | − |
| Cu | +0.33 | − | Cu | Cu " (?). | − | | − | − |
| Brass | | − | Brass. | − | − | − | − | − |
| Hg (Hg₂ / Hg) | +0.77 / +0.83 | − | Hg | Not tried. | − | − | Not tried. | Not tried. |
| Ag | +0.80 | − | Ag | Ag faint. | − | − | | − |
| Pd | | − | Pd | Pd " | − | − | − | − |
| Pt | +0.86 | − | Pt | Pt " | − | − | − | − |
| Au | +1.08 | − | Au | Au " | − | − | − | |
| C | | − | C | C " (?). | − | − | − | − |

is possibly due to formation of a resistant film of oxide over the surface of the chromium.

In the above experiment with Zn-Pt couples we really have an example of electrochemical action at a distance. The Zn and the Pt can be placed in separate containers connected by a salt bridge. The Zn is surrounded by NaCl solution and the Pt by NaCl-oxyluciferin-luciferase-oxygen solution. No luminescence is to be observed in either container until the Zn and Pt are connected by a wire, when the Pt surface will luminesce beautifully.

It should be possible to construct another type of oxidation-reduction cell in which the reduction of oxyluciferin takes place at a distance, but using the same metal for each electrode, and, as a reducing agent, a solution which will cause reduction when mixed directly with oxyluciferin. The above can be realized by the following device similar to Ostwald's well known liberation of iodine from KI (oxidation) by Br water at a distance.

A vessel, *A*, is filled with oxyluciferin-luciferase-NaCl solution and a Pt electrode; another vessel, *B*, is filled with $Na_2S$ solution and a Pt electrode. A salt bridge connects the two. No luminescence is to be observed until the two Pt electrodes are connected by a metallic conductor. Then the oxyluciferin in *A* is reduced in contact with the electrode and luminesces when oxidized again, just beyond the electrode layer. The equation representing the change is

$$L + 2\,HOH + Na_2S = LH_2 + 2\,NaOH + S$$

Sulfide ions give up negative charges at the anode (oxidation), while H ions lose positive charges at the cathode and reduce the oxyluciferin.

It is also possible to reduce oxyluciferin in presence of molecular hydrogen provided that a catalyst is present. As is well known (Maxted, 1919; Rideal and Taylor, 1919), there are two groups of catalysts used in hydrogenation processes in the industries; namely, the Pt (Pt, Pd, Ru, Ro, Ir, Os) group and the Ni (Ni, Fe, Co, Cu) group.

Pt and Pd are active examples of the first group. A palladinized surface, if saturated with hydrogen and then placed in oxyluciferin-luciferase-oxygen solution, will glow beautifully. No luminescence appears if the palladinized surface is first exposed to oxygen. A

continuous luminescence appears if hydrogen from a cylinder is bubbled through an oxyluciferin-luciferase solution which contains palladinized gauze. It is not necessary that the hydrogen be in the atomic or nascent condition in contact with the palladium. Molecular hydrogen in contact with palladium is converted into the active state as is evidenced by the luminescence in the above mentioned experiment. We may therefore conclude that the reason palladium acts as a catalyst is because of its power to convert molecular into atomic hydrogen.

It may perhaps be noted in passing that we have here a means of producing a luminescent lamp which would burn for an indefinite time provided a slow stream of hydrogen and oxygen be supplied.

A large palladinized surface would continually reduce oxyluciferin which would just as continually reoxidize with luminescence in the presence of luciferase and oxygen. The reactions are

$$L + H_2 + Pd = LH_2 + Pd$$
$$LH_2 + O + \text{luciferase} = H_2O + L + \text{luciferase}$$

Crossing out the members on each side of the two equations, we see that the permanent chemical change is the union of $H_2 + O$ to form water. Pd and luciferase are catalysts. So long as atomic hydrogen and oxygen are present, we should have this double cycle of events occurring and luminescence would cease only when secondary changes in the luciferin, luciferase, or palladium occurred.

It is a well known fact that hydrogenations involve the production of heat. In the hydrogenation of some oils, artificial cooling must be resorted to. In the reverse process of dehydrogenation heat is absorbed. The equilibrium equations for oxidation of luciferin might be represented thus

$$LH_2 \rightleftarrows L + H_2 - x \text{ Cal.}$$
$$H_2 + O \rightleftarrows H_2O + 58.3 \text{ Cal.}$$

The process would be similar to the oxidation of an alcohol

$$CH_3CH_2OH \rightleftarrows CH_3CHO + H_2 - 11.5 \text{ Cal.}$$
$$H_2 + O \rightleftarrows H_2O + 58.3 \text{ Cal.}$$

According to Rideal (1921) the heat absorbed during dehydrogenation of ethyl alcohol is 11.5 Cal. per gram mol. Where one reaction is endothermic and the other exothermic, it is quite possible for the

heat produced nearly to balance the heat absorbed, and consequently for very little temperature change to accompany the process. No definite temperature change has as yet been demonstrated to accompany animal luminescence.

Another method of producing a continuous luminescence is to place a palladinized surface in contact with oxyluciferin + luciferase + $NaHPO_2$. The sodium hypophosphite acts as an oxygen accepter. The Pd under these conditions can decompose water. The oxygen of water is taken by the $NaHPO_2$ which forms $NaHPO_3$ while the $H_2$ reduces the oxyluciferin. Under these conditions the luminescence should last as long as there is any $NaHPO_2$ and oxygen left in solution.

It is very likely that living organisms whose luminescence is intracellular, utilize their store of luciferin over and over again. While it has been tacitly assumed that luminous bacteria, for instance, continually produce luciferin, which is oxidized to some waste product and excreted, we may quite as logically believe that luciferin is oxidized in one part of the cell and oxyluciferin reduced in another, these processes forming a continuous cycle, such as may be imitated under the chemical conditions previously discussed. There is a growing conviction that in some regions of the cell oxidations predominate, in other regions reductions occur. If we may assume that such conditions exist in the photogenic cell, another marvel of economy in the living world is presented. Not only is living light most efficient in its physical make-up, containing only those wave-lengths which affect the eye, but also most economical in the utilization of chemical material, the organism employing a reversible luminescent reaction, whose equilibrium may be shifted in one or the other direction in contiguous regions of the same cell.

## SUMMARY.

Oxyluciferin may be reduced to luciferin at cathodes, when an electric current is passed through the solution, or at cathodes formed by metal couples in solution, or at cathodes of oxidation-reduction cells of the $NaCl - Pt - Pt - Na_2S$ type. It is also reduced at those metal surfaces (Al, Mn, Zn, and Cd) which liberate nascent hydrogen from water, although no visible hydrogen gas separates from the surface. Molecular hydrogen does not reduce oxyluciferin even though

very finely divided but will reduce oxyluciferin in contact with palladium. Palladium has no reducing action except in presence of hydrogen, and apparently acts as a catalyst by virtue of some power of converting molecular into atomic hydrogen. Conditions are described under which a continuous luminescence of luciferin can be obtained. This luminescence may be used as a test for atomic hydrogen. It is suggested that the steady luminescence of bacteria is due to continuous oxidation of luciferin to oxyluciferin and reduction of oxyluciferin to luciferin in different parts of the bacterial cell.

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# ON THE GROWTH AND RESPIRATION OF SULFUR-OXIDIZING BACTERIA.*

By SELMAN A. WAKSMAN and ROBERT L. STARKEY.

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(Received for publication, October 23, 1922.)

## INTRODUCTION.

The respiration of sulfur-oxidizing bacteria is of particular interest, since this group of organisms belongs to the very few forms of life (autotrophic) which are able to obtain the carbon from the reduction of the $CO_2$ of the atmosphere and the necessary energy from the oxidation of inorganic substances, in this case sulfur compounds; in other words, it is able to assimilate carbon chemosynthetically, using inorganic sulfur and sulfur compounds as a source of energy. In the case of this group of organisms, accurate information can be obtained on the amount of chemical energy required in the process of building up complex organic substances from minerals and carbon dioxide. The simple composition of the nutrients will also help to throw light on the differentiation between the structural and functional energy required in metabolism as well as on other disputed questions of metabolism, such as the influence of concentration of nutrients, neutralization of waste products, and amount of total available energy utilized by the organism in connection with the carbon assimilation.

Respiration in the study of autotrophic bacteria is comparable with respiration of all the other forms of life in that it results in the liberation of energy necessary for the life activities of the organism. Respiration is commonly understood to mean the liberation of carbon dioxide as a result of the oxidation of organic matter by the organism, the addition of oxygen following a series of enzymatic reactions. In the case of sulfur oxidation, the energy is liberated as a result of direct combustion of the sulfur, as pointed out in 1887 by Winogradsky.

The recent investigations on the autotrophic bacteria brought out the fact that only very few organisms are strictly autotrophic; here belong the nitrifying bacteria, as pointed out by Winogradsky and others, particularly in the careful work of Meyerhof on the respiration and energy transformation of these organisms, and the sulfur organism under consideration. The methane, hydrogen, and iron bacteria are facultative autotrophic, or can exist also heterotrophically, as

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shown by Niklewski, Lieske (1912), and Beijerinck (1910). Of the sulfur bacteria, we know that at least some organisms, like the *Sulfomonas* (*Thiobacillus*) *denitrificans* of Beijerinck (1920) and the related form of Trautwein, can also exist heterotrophically. In other words, some forms are facultative autotrophic, or are capable of obtaining their energy both chemosynthetically and from organic matter. *Sulfomonas denitrificans* will oxidize sulfur compounds, using nitrate nitrogen, which results in the oxidation of the sulfur and the reduction of the nitrate (denitrification); about 1 Cal. is produced per gram of decomposed nitrate (Beijerinck (1920)).

The organism used in these experiments, *Sulfomonas* (*Thiobacillus*) *thiooxidans*, was isolated from sulfur floats (rock phosphate) soil composts, in which it is chiefly responsible for the oxidation of the elementary sulfur to sulfuric acid. It has been grown for nearly 2 years in inorganic media of simple composition, with elementary sulfur as the only source of energy, and it has not decreased in vitality and power of sulfur oxidation.

*Sulfomonas thiooxidans* will derive its energy from the oxidation of sulfides, thiosulfates, and primarily of elementary sulfur to sulfuric acid. It derives its carbon from carbon dioxide in the atmosphere, although it is not injured by the presence of organic matter and bicarbonates. The optimum reaction is distinctly acid, equivalent to pH 3.0 to 4.0. The presence of carbonates tending to make the medium alkaline are, therefore, injurious to the growth of the organism. Bicarbonates, particularly in the presence of sufficient buffering substances, like phosphates, may not be injurious. It has been generally assumed that the nitrifying bacteria derive their carbon from carbonates and bicarbonates. Meyerhof has demonstrated, however, that the presence of these substances is of importance, because they serve as buffers to keep the medium alkaline, since the optimum reaction for nitrifying bacteria lies at a pH of 8.0 to 9.0. When phosphates are substituted in place of carbonates, those bacteria are found to derive their carbon from carbon dioxide. The *Sulfomonas thiooxidans* having its optimum at a distinctly acid range will thrive very well in the complete absence of all traces of carbonates or bicarbonates. Like the nitrifying bacteria, it does not grow on organic media, unless sulfur is present as a source of energy, and is thus found to be unable to derive its energy from any organic materials. In this respect the facultative autotrophic *Sulfomonas denitrificans* studied by Beijerinck (1920) and Trautwein behaves differently since it is able to grow also on common organic media.

Winogradsky was the first to investigate the metabolism of the sulfur bacteria without, however, succeeding in isolating the organisms in pure culture. When a culture of *Beggiatoa* (thread-forming, colorless organism) is grown in a medium containing hydrogen sulfide, it is found to accumulate sulfur within its cells as a result of the oxidation of $H_2S$ to $H_2O$ and S. When left in the medium free from hydrogen sulfide, the sulfur granules present in the threads are found to disappear within 24 hours, presumably due to the oxidation of the sulfur to sulfuric acid; the medium, however, never became acid. This was explained by

the fact that the impure water was saturated with calcium carbonate, which neutralized the resulting acid. During the same period, the bacterial mass is almost doubled. Kruse calculated, from the data obtained by Winogradsky, that for the assimilation of 1 gm. of carbon, 8 to 19 gm. of sulfur have to be utilized. The reaction takes place as follows:

$$H_2S + O = H_2O + S$$
$$S + 3O + H_2O = H_2SO_4 + (142.3\ Cal.)$$

Kruse suggested then that one atom of sulfur could liberate more energy than would be necessary for the building up of three atoms of carbon, or to synthesize aminopropionic acid from an equal weight of propionic acid and one atom of nitrogen (in the form of nitric acid).

As to the purple bacteria, it has been definitely demonstrated by Molisch that they are unable to assimilate carbon dioxide with a splitting of oxygen and that they thrive best in highly concentrated organic media.

Keil found that *Beggiatoa* and *Thiothrix* can exist in media entirely free from organic nitrogen, but are not injured by organic substances in not too high concentrations, with $CO_2$ as the only source of carbon; sufficient amounts of carbonates of alkali earths should be present to neutralize the acid formed from the oxidation of the sulfide.

The colorless sulfur bacterium isolated by Nathansohn and later described by Beijerinck as *Thiobacillus thioparus* (*Sulfomonas thioparus*) was found to be able to derive its carbon both from carbonate and from carbon dioxide of the atmosphere. This organism oxidizes thiosulfates and sulfide; the medium was not found to become acid in the absence of carbonates. This was explained by the fact that the thiosulfate is oxidized to sulfate and free sulfur and the sulfides to free sulfur.

$$Na_2S_2O_3 + O = Na_2SO_4 + S$$
$$H_2S + O \quad = H_2O + S$$

This organism was found by Nathansohn to be able to obtain its carbon from carbonates. Trautwein, however, who studied a closely related organism which could use thiosulfate as the only source of energy, found that it obtained its carbon from carbon dioxide of the atmosphere or in solution, in the form of bicarbonates, but not from carbonates. This organism could also obtain its energy from organic compounds; in other words, it could exist both under autotrophic or heterotrophic conditions. The thiosulfate was oxidized to sulfate and higher sulfates, without an increase in the acidity of the medium and without sulfur separation. Beijerinck (1920) suggested that here we have an hereditary modification as a result of great changes in the nature of the food. Lieske (1912) found the denitrifying sulfur-oxidizing form capable of utilizing the carbonates of ammonium, calcium, magnesium, manganese, and sodium as sources of carbon, while free carbonic acid was not assimilated due to the high acidity of the medium. $Na_2S_2O_3$, $Na_2S_2O_6$, $K_2S$, $H_2S$, and finely divided sulfur can be used as sources of energy. For every gram of carbon assimilated, 100 gm. of $Na_2S_2O_3 \cdot 5H_2O$ are

oxidized. In the presence of an excess of nitrate the sulfur compounds were completely oxidized to sulfate. The sulfur was found to be first oxidized to lower acids, then to sulfuric acid.

## EXPERIMENTAL.

*Culture.*—The organism used in the following investigations (*Sulfomonas thiooxidans*) is a minute, non-spore-forming, rod-shaped to oval, unicellular, colorless organism, measuring about 0.5 to 1 micron long and about 0.5 micron wide, described in detail elsewhere (Lipman, Waksman, and Joffe (1921), and Waksman and Joffe (1922, *b*)). It oxidizes elementary sulfur quantitatively to sulfuric acid; thiosulfates are completely oxidized to sulfates, without the secretion of elementary sulfur. It acts also, but to a lesser extent, on sulfides, oxidizing them to sulfates.

The medium of the following composition was used:

| | |
|---|---|
| $(NH_4)_2SO_3$ | 0.2 gm. |
| $MgSO_4$ | 0.1–0.5 " |
| $FeSO_4$ | 0.01 " |
| $CaCl_2$ | 0.25 " |
| $KH_2PO_4$ | 3.0–5.0 " |
| Powdered sulfur | 10.0 " |
| Distilled water | 1,000.0 cc. |

In the experiments on carbon assimilation, the water was distilled twice, the second time with glass containers and condensers only, in a manner similar to that used for preparing conductivity water. The sulfur is weighed out separately into the individual flasks, previously cleaned with potassium bichromate and sulfuric acid. The medium is placed, in 100 cc. and 300 cc. quantities, in 250 cc. and 1 liter Erlenmeyer flasks respectively, which are then plugged with cotton and sterilized in flowing steam, for 30 minutes, on 3 consecutive days, and all manipulations of the culture were carried out under sterile conditions. Sterility is not necessary with a well growing culture, since the medium will not allow any appreciable development of other microorganisms. Transfers were always made by means of a sterile platinum loop or sterile pipette, into fresh sterile flasks of medium or into the containers for analysis. A vigorously growing 7 day old culture was always used for inoculating the experimental flasks. The cultures were incubated at 26–28°C. for the desired length of time.

*Methods of Analysis.*—The reaction is determined both by the method of titration and by the hydrogen ion concentration. In titrating, 5 or 10 cc. of the culture were titrated with 0.1 N NaOH, using phenolphthalein as an indicator. Titer designates the amount of 0.1 N NaOH required to neutralize 1 cc. of the culture. The hydrogen ion concentration was determined by means of the Clark and Lubs series of indicators and checked carefully by an electrometric apparatus, consisting of a modification of the Hildebrand apparatus, in which the Clark vessel is used. However, at very high hydrogen ion concentrations (at pH less

than 1.0), which are readily obtained in the culture of the organism, the titer was found to be a more reliable index of the amount of acid formed, since both the colorimetric and electrometric methods for determining the H ion concentrations were found to be not sensitive enough with the apparatus available. The sulfates were determined by precipitating an aliquot filtered portion of the culture with $BaCl_2$, washing and weighing the $BaSO_4$; the residual sulfur was obtained by filtering the culture carefully through a weighed Gooch crucible, washing with a large amount of slightly acidulated water and drying to constant weight.

The total carbon of the culture was determined according to the following procedure. A wet combustion apparatus, similar to the one described by Gortner and to the one used by Meyerhof, was used (Fig. 1). A set of KOH bulbs, *A*, containing a 1:1 solution of

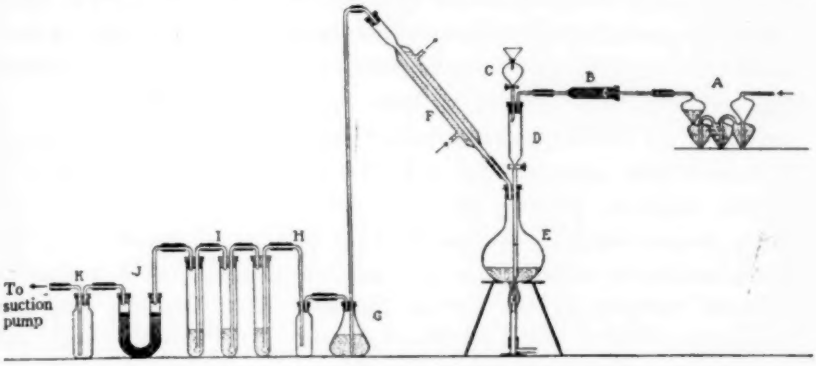


FIG. 1. Apparatus for carbon determinations.

KOH and a soda-lime tube, *B*, served to remove all $CO_2$ from the entering air. 100 cc. of the unfiltered mixed culture were introduced into a 500 cc. Pyrex flask, *E*, where the digestion took place. Concentrated sulfuric acid was added through the separatory funnel, *C*, into the wide mouthed separatory funnel, *D*, through which it entered into *E*. The condenser, *F*, led to a 250 cc. Erlenmeyer flask, *G*, containing a saturated solution of $Ag_2SO_4$ in 5 per cent $H_2SO_4$. This solution served to remove the chlorides from the gas. The bottle, *H*, is a trap to catch any of the standard alkali solution in case of back suction. The absorption of the $CO_2$ was carried out in *I*, consisting of a series of three large hard glass test-tubes of about 70 cc. capacity, each containing 25 cc. of standardized approximately

0.05 N $Ba(OH)_2$. The glass tubes through which the gas was passing were provided at the bottom with perforated bulbs similar to the Folin aeration tubes. *J* contained soda-lime to prevent any reversed diffusion of $CO_2$. *K* is a trap to catch any water from the water pump in case of back suction due to lowered water pressure.

The determinations were carried out as follows: A 100 cc. portion of the solution to be analyzed was placed in the combustion flask and 2.5 gm. $KMnO_4$ were added. After attaching the flask, suction was applied and 50 cc. of concentrated $H_2SO_4$ were very slowly admitted through the separatory funnel *D*. It took about half an hour to add this acid, as considerable heat was generated accompanied by a rather rapid evolution of gas. The stop-cock at *D* was then closed and the solution heated to gentle boiling with a small flame. After the gas had practically all been driven from the flask, as indicated by a backing up of the solutions, the stop-cock, *D*, was opened and the apparatus aerated without heat for about 30 minutes. A stream of air could be drawn rather rapidly through the apparatus. Heat was then again applied with the stop-cock closed, followed by further aeration; the two processes were then again repeated. The whole determination required about 3 hours. This period of time was found to be sufficient for the complete combustion of the carbon and the removal of the carbon dioxide. The $Ba(OH)_2$ was then titrated back with standard, approximately 0.05 N oxalic acid, with phenolphthalein as an indicator. The solutions were standardized daily. Two duplicate determinations were always made on the same culture.

The accuracy of the method was checked up by determining the carbon content of saccharose and urea, whereby 95 to 98 per cent of the theoretical amount of carbon was obtained.

### *Chemistry of Sulfur Oxidation.*

By determining the sulfur and sulfates in the inoculated and uninoculated flasks, it is found that the sulfur is almost quantitatively transformed into sulfuric acid, as shown in Table I. In all cases, the amount of sulfur obtained in the form of sulfate was found to be greater than the loss in elementary sulfur unoxidized, due probably

to some inaccuracy in figuring the loss of moisture during the incubation period.

This would lead us to conclude that the sulfur is oxidized, by the microorganism in question, according to the following reaction, without any intermediary reactions taking place.

$$S + 3O + H_2O = H_2SO_4$$

When thiosulfate is used as a source of energy, it is found to be oxidized completely to sulfates, with a corresponding increase in acidity. By using 5 gm. of $Na_2S_2O_3 \cdot 5H_2O$ per liter and incubating the cultures for 30 days, the reaction of the culture changed from pH

TABLE I.

*Oxidation of Elementary Sulfur to Sulfuric Acid by Sulfomonas thiooxidans.*

| Incubation. | Amount of culture in flask. | Control flask. | | Inoculated flasks. | | Elementary sulfur disappeared. | Increase in sulfate. |
|---|---|---|---|---|---|---|---|
| | | Sulfur. | Sulfate.* | Sulfur. | Sulfate. | | |
| *days* | *cc.* | *mg. of S* | *mg. of S* | *mg. of S* | *mg. of S* | *mg. of S* | *mg. of S* |
| 15 | 100 | 1,001 | 86.4 | 788 | 302.1 | 213 | 215.7 |
| 30 | 100 | 992 | 90.5 | 735 | 354.0 | 257 | 263.5 |
| 15 | 300 | 3,002 | 112.2 | 2,496 | 633.0 | 506 | 520.8 |
| 30 | 300 | 2,997 | 126.5 | 1,974 | 1,168.0 | 1,023 | 1,041.5 |

* Mg. of soluble sulfates as sulfur in flask; averages of 3 flasks are given; the concentration of sulfates in the small flasks was greater, due to the fact that, in these, a medium containing 2 gm. $(NH_4)_2SO_4$ and 0.5 gm. $MgSO_4$ per liter was used.

5.0 to pH 1.8, the thiosulfate completely disappeared, and the sulfate increased from 12.60 mg. of S as sulfate in 100 cc. of medium to 142.73 mg.; in other words, all the thiosulfate was transformed into sulfate (the theoretical content of total sulfur in 100 cc. of medium is 141.6). The reaction should, therefore, be written as follows:

$$Na_2S_2O_3 + 4O + H_2O = Na_2SO_4 + H_2SO_4 \qquad (1)$$

It may be recalled, in this connection, that the sulfur organism of Nathansohn did not change the reaction to acid. He therefore suggested for his organism the following reaction:

$$3\,Na_2S_2O_3 + 5O = 2\,Na_2SO_4 + Na_2S_4O_6 \qquad (2)$$

The reaction did not account for the precipitation of sulfur by the organism from the thiosulfate. Beijerinck therefore suggested the following reaction:

$$Na_2S_2O_3 + O = Na_2SO_4 + S \qquad (3)$$

The organism of Trautwein oxidized thiosulfate without the separation of sulfur, as follows:

$$7\ Na_2S_2O_3 + 17O = 2\ Na_2SO_4 + Na_2S_4O_6 + 4\ Na_2S_2O_6 \qquad (4)$$

The difference in the four reactions will account for the fact that different organisms were used by the various workers and that the organism under consideration (*Sulfomonas thiooxidans*) did not precipitate any sulfur and caused a definite increase in acidity; the organism of Nathansohn and Beijerinck (*Sulfomonas thioparus*) caused an intense precipitation of free sulfur and no increase in acidity; the organism of Trautwein (*Sulfomonas denitrificans* var.) caused no precipitation of sulfur and no acid formation.

### *Carbon Assimilation.*

*Qualitative.*—Sterile 100 cc. portions of the regular medium in 250 cc. Erlenmeyer flasks were inoculated as usual and placed under bell jars, sealed around with a mixture of rubber and paraffin to prevent any leakage. The air aspirated through the jars was passed first through soda-lime tubes, then through a 50 per cent solution of KOH to free the air from carbon dioxide. Some flasks received 0.1 per cent $NaHCO_3$ and others 0.1 per cent dextrose. A duplicate series of flasks were placed outside of the apparatus on the incubator shelves. After 5 days incubation, the amount of sulfur oxidized in the various flasks, as shown by change in reaction, was determined. The results are presented in Table II.

It can be seen from the above experiments that the organism does not grow in a $CO_2$-free atmosphere and it cannot derive its carbon from dextrose. The slight increases in acidity are probably due to the acid introduced with the inoculum and perhaps imperfect evacuation of chamber the first day. In the presence of bicarbonate, the oxidation of sulfur is greatly reduced due to the unfavorable reaction, but there was a definite amount of growth and sulfur oxidation in

the $CO_2$-free atmosphere. This experiment was repeated again and it was definitely established that *Sulfomonas thiooxidans* can derive its carbon only from carbon dioxide of the atmosphere but not from organic materials; in the presence of carbon dioxide in solution, and in the form of bicarbonates, growth will also take place if the reaction is favorable for the activities of the organism.

*Quantitative.*—300 cc. portions of the medium and 3 gm. of sulfur were placed in a series of 1 liter flasks, sterilized and inoculated as usual. Some of the flasks were uninoculated and left as controls. At the end of various periods of incubation, one or two flasks were taken out for analysis. Two 100 cc. portions of the culture from each flask were used for duplicate carbon determinations; the rest of the

TABLE II.

*Influence of $CO_2$ upon the Growth of Sulfomonas thiooxidans.*

| Control. | Ordinary atmosphere. | | | | | | $CO_2$-free atmosphere. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Regular medium. | | 0.1 per cent $NaHCO_3$ | | 0.1 per cent dextrose. | | Regular medium. | | 0.1 per cent $NaHCO_3$ | | 0.1 per cent dextrose. | |
| | pH | Titer. | pH | Titer. | pH | Titer. | pH | Titer. | pH | Titer. | pH | Titer. |
| Uninoculated..... | 4.4 | 0.23 | 6.8 | 0.12 | 4.4 | 0.23 | 4.4 | 0.23 | 6.8 | 0.12 | 4.4 | 0.23 |
| Inoculated... | 1.4 | 1.05 | 2.8 | 0.36 | 1.5 | 0.92 | 3.8 | 0.25 | 6.0 | 0.20 | 3.3 | 0.25 |

culture was filtered through analytical filter paper; two 25 cc. portions of the filtrate were used for sulfate determinations, and the rest was used for titration and determination of the hydrogen ion concentration. The results are tabulated in Table III.

The course of sulfur oxidation and carbon assimilation is uninterrupted. Nearly one-third of the sulfur present in the medium is oxidized in 42 days, with the reduction of the hydrogen ion concentration of the medium to a pH of 1.0 or less and with the formation of titrable acidity equivalent to fourth-normal (0.25 N) acid. The initial titration of the control is due to the buffer content of the medium (5 gm. $KH_2PO_4$ per liter). The ratio of the sulfur oxidized to the carbon assimilated varies from 28.0 at the beginning of the active growth period to 34.22, falling down to 31.25, at the end of the experiment, with an average of 31.82 parts of sulfur oxidized for

TABLE III.

*Course of Sulfur Oxidation and Carbon Assimilation.*

| Age of culture. | Total carbon, as mg. of C in 100 cc. | | | | Total sulfate, as mg. of S in 100 cc. | | | | $\frac{S^*}{C}$ | pH | Titer 1 cc. = cc. of 0.1 N NaOH. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | Average. | Blank subtracted. | 1 | 2 | Average. | Blank subtracted. | | | |
| | | | | | Uninoculated. | | | | | | |
| *days* | | | | | | | | | | | |
| 0 | 2.318 | 1.885 | 2.102 | | 9.51 | 9.67 | 9.59 | | | 4.0 | 0.40 |
| 13 | 2.349 | 1.913 | 2.131 | | 11.19 | 11.27 | 11.23 | | | 3.9 | 0.41 |
| 43 | 2.167 | 2.087 | 2.127 | | 11.60 | 11.96 | 11.78 | | | 4.0 | 0.42 |
| Average ..... | | | 2.120 | | | | 10.87 | | | 4.0 | 0.41 |
| | | | | | Inoculated. | | | | | | |
| 5 | 4.725 | 4.680 | 4.703 | 2.583 | 83.272 | 84.320 | 83.296 | 72.426 | 28.04 | 2.0 | 0.90 |
| 9 | 5.201 | 5.201 | 5.201 | 3.081 | 111.214 | 111.274 | 111.244 | 100.374 | 32.58 | 1.5 | 1.04 |
| 12 | 6.202 | 6.754 | 6.478 | 4.358 | 160.016 | 160.016 | 160.016 | 149.146 | 34.22 | 1.3 | 1.37 |
| 19 | 8.917 | 9.606 | 9.262 | 7.142 | 253.150 | 253.202 | 253.176 | 242.306 | 33.94 | 1.2 | 2.02 |
| 21 | 10.164 | 9.405 | 9.785 | 7.665 | 252.062 | 252.098 | 252.080 | 241.210 | 31.47 | 1.2 | 2.00 |
| 27 | 11.156 | 11.857 | 11.507 | 9.387 | 303.842 | 303.788 | 303.815 | 292.945 | 31.21 | 1.2(?)† | 2.20 |
| 42 | 11.528 | 12.389 | 11.959 | 9.839 | 318.376 | 318.404 | 318.390 | 307.520 | 31.25 | 1.0(?)† | 2.50 |
| Average ..... | | | | | | | | | 31.82 | | |

$\frac{^*S}{C}$ = ratio of sulfur oxidized to carbon assimilated.

† Determination inaccurate due to high acidity.

every gram of carbon assimilated from the carbon dioxide of the atmosphere. The somewhat low initial ratio may be due to the fact that in the early period of growth, when carbon assimilation is most active, the energy obtained is utilized most economically.

It is interesting to note in this connection that the $\frac{N}{C}$ ratio for nitrite-forming bacteria (Meyerhof) was found to be 35, while for nitrate bacteria 135, but in view of the fact that the heat liberated in the second process is about one-fourth of that liberated in the first,

TABLE IV.

*Assimilation of Carbon by Sulfomonas thiooxidans with Thiosulfate as a Source of Energy.*

| | Total carbon, mg. of C in 100 cc. | | Total sulfate, mg. of S in 100 cc. | | $\frac{S}{C}$ | pH | Titer. |
|---|---|---|---|---|---|---|---|
| | Determinations. | Average. | Determinations. | Average. | | | |
| Control. | 1.663 | | 11.37 | | | | |
| | 1.998 | | 12.85 | | | | |
| | 1.705 | 1.789 | 16.70 | | | | |
| | | | 14.55 | 13.87 | | 4.9 | 0.14 |
| Inoculated. | 3.577 | | 130.98 | | | | |
| | 3.284 | | 130.49 | | | | |
| | 3.965 | | 135.38 | | | | |
| | 3.764 | 3.648 | 135.87 | 133.18 | | 1.85 | 0.53 |
| Control subtracted. | | 1.859 | | 119.31 | 64.2 | | |

the utilization of energy, in both processes, for chemosynthetic purposes, is identical.

With $Na_2S_2O_3$ as a source of energy, Lieske (1912) found that 10.9 mg. of carbon were assimilated by *Sulfomonas denitrificans* for every gram of thiosulfate oxidized; in other words, the oxidation of 405 mg. of sulfur in the form of thiosulfate will allow an assimilation of 10.9 mg. of carbon chemosynthetically, with $\frac{S}{C} = 37.2$. For the study of the utilization of thiosulfate as a source of energy by *Sulfomonas thiooxidans*, 5 gm. of $Na_2S_2O_3 \cdot 5H_2O$ were used, in place of the 10 gm. of sulfur, per liter of medium. The flasks were incubated for 16 to 20 days. The results are recorded in Table IV.

† Determination inaccurate due to high acidity.

The theoretical amount of sulfur as thiosulfate in the medium is 129.0 mg. in 100 cc.; 119.31 mg. were found in the form of sulfate. The higher sulfur-carbon ratio is due in this case to the fact that there is less energy liberated from the oxidation of one unit of sulfur in the form of thiosulfate than in the form of elementary sulfur.

*Heat Formation and Sulfur Oxidation.*—As pointed out above, the oxidation of sulfur by *Sulfomonas thiooxidans* takes place according to the following reaction:

$$\underset{}{S} + \underset{}{30} + \underset{68.5}{H_2O} = \underset{210.8}{H_2SO_4} + 142.3 \text{ Cal.}^1$$

In other words, from the oxidation of 1 mol or 32 gm. of sulfur, 142.3 Cal. will be liberated. It takes 113 Cal. to transform 1 mol of $CO_2$ into dextrose; or to assimilate 12 gm. of carbon, 113 Cal. are required. But in the process of assimilation of 12 gm. of carbon, 31.82 times as much sulfur has to be oxidized, or $31.82 \times 12 = 381.84$ gm. This quantity of sulfur will liberate $\frac{381.84}{32} \times 142.3 = 1{,}698$ Cal. Of this total heat liberated, only 113 Cal., or 6.65 per cent, are utilized for the assimilation of carbon.

The percentage of energy made available from the oxidation of sulfur and utilized by the organism for chemosynthetic purposes, *i.e.* carbon assimilation from the $CO_2$ of the atmosphere, is found to be much higher than in the case of higher plants, which are variably estimated to utilize 0.5 to 3 per cent of the energy of rays of the sun. The much more favorable utilization of the energy for the chemosynthetic carbon assimilation than for the photosynthetic has also been pointed out for nitrifying bacteria by Meyerhof, who found that both the nitrite and nitrate formers utilize about 5 per cent of the energy liberated for the chemosynthetic assimilation of carbon.

The calorific quotient of oxygen is found, in the sulfur oxidation, to be $\frac{141.5}{3 \times 16} = 2.95$; or for every milligram of oxygen consumed in the reaction, 2.95 cal. of heat are liberated. In this case it approaches the calorific quotient of oxygen in the combustion of organic substances which is 3.0 to 3.5 cal. for 1 mg. of oxygen and is much higher than

[1] Data are taken from Landolt-Börnstein tables, 4th edition.

that obtained in nitrite and nitrate formation, which is only about 1.35 to 1.75 cal.

*Concentration of Sulfates and Sulfur Oxidation.*—Various investigators (Meyerhof) called attention to the fact that the accumulation of the oxidation product by nitrite and nitrate bacteria injures growth of the organisms and respiration. Trautwein could not demonstrate any injurious effect of sulfate upon the growth of sulfur-oxidizing bacteria; even where as much as 5.0 per cent $Na_2SO_4$ has been used, no injurious influence of the oxidation product was found. NaCl,

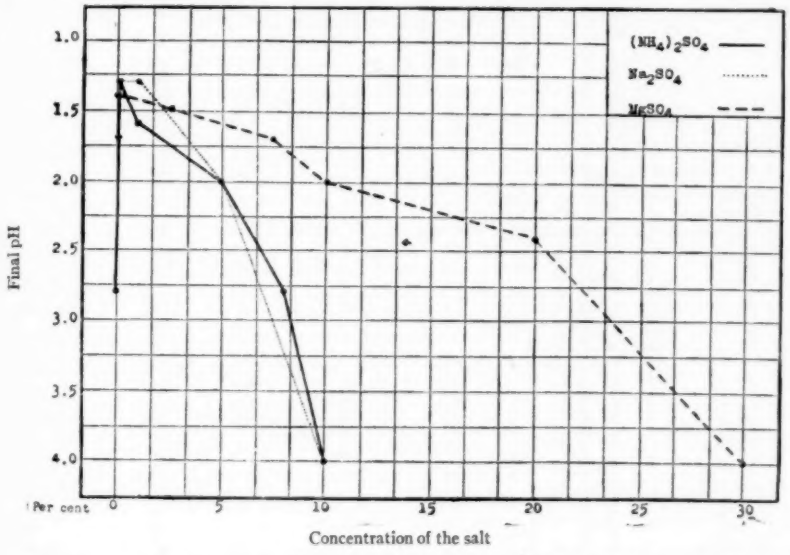


FIG. 2. Effect of concentration of Na, ($NH_4$), and Mg sulfates on sulfur oxidation by *Sulfomonas thiooxidans*.

however, had an injurious influence, which was explained by the influence of the Cl-ion.

Three different sulfates, $MgSO_4$, $Na_2SO_4$, $(NH_4)_2SO_4$ (the influence of sulfuric acid is reported later), have been tested as to their influence on sulfur oxidation by *Sulfomonas thiooxidans*, and in no case could the injurious influence be ascribed to the sulfate-ion, growth coming to a standstill only with very high concentrations. Where an influence has been found, it was due more to the influence of the cation and not to the oxidation product, as seen from Fig. 2. Calcium sulfate was found to be entirely uninjurious to the growth of the organism;

TABLE V.

*Influence of Salt Concentration upon Sulfur Oxidation by Sulfomonas thiooxidans.*

| Concentration. | 0 | | 0.01 | | 0.1 | | 1 | | 2.5 | | 5 | | 10 | | 20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH | S* | pH | S | pH | S | pH | S | pH | S | pH | S | pH | S | pH | S |
| *per cent* | | | | | | | | | | | | | | | | |
| $KH_2PO_4$ | 1.0 | 147.17 | | | 1.2 | 181.15 | | | 2.8 | 49.63 | 4.0 | | 4.0 | 0 | | |
| $MgSO_4$ | 1.2 | 282.8 | | | 1.2 | | 1.2 | 271.8 | | | 1.6 | 205.5 | 1.3 | 280.9 | 1.9 | 41.1 |
| $(NH_4)_2SO_4$ | 2.8 | | 1.7 | | 1.3 | | 1.6 | | | | 2.0 | | | | | |
| $Na_2SO_4$ | 1.2 | 336.4 | | | | | 1.2 | 300.8 | | | 1.4 | 112.4 | 2.0 | 35.2 | | |
| $NH_4Cl$ | | | | | 1.0 | 249.9 | | | 1.4 | 114.5 | | | 4.0 | 0 | 4.0 | |
| NaCl | 1.2 | 260.3 | | | | | 1.2 | 277.3 | | | 1.6 | 75.6 | 4.0 | 0 | 4.0 | |
| $MgCl_2$ | 1.2 | 252.0 | | | | | 1.0 | 310.6 | | | 1.2 | 162.7 | 1.8 | 34.4 | 4.0 | |
| $NaNO_3$ | | | 1.3 | 112.4 | 1.3 | 110.4 | 2.6 | 10.3 | 3.8 | 0 | 3.8 | 0 | | | | |
| $Ca(NO_3)_2$ | | | 1.3 | 118.9 | 1.3 | 102.1 | 2.6 | 12.4 | 3.6 | 0.45 | 3.8 | 0 | | | | |
| $KAl(SO_4)_2$ | | | 1.2 | | 1.2 | | | | 1.4 | | | | | | | |

* S = milligrams of sulfur oxidized to sulfate in 100 cc. of medium; pH of control medium = 4.0; cultures incubated 7 days.

in view of the fact that this salt is soluble only in small amounts, large amounts of it can be added without appreciable injury to the culture.

To compare the influence of sulfates and other anions as well as cations upon the oxidation of sulfur, a series of experiments was carried out, and the results are summarized in Tables V and VI. The results indicate that, among the anions, the sulfate and chloride are uninjurious in low concentrations and become injurious only in concentrations of 0.2 to 0.5 molar, the nitrate being most toxic. The phosphates seem to become toxic in lower concentrations than

TABLE VI.

*Salt Concentrations Which Limit Growth of Sulfomonas thiooxidans.*

| | Molar Concentration. | |
|---|---|---|
| | No growth. | Growth. |
| $H_2SO_4$ | 1.00 | 0.50 |
| $MgSO_4$ | 1.20 | 0.80 |
| $(NH_4)_2SO_4$ | 0.80 | 0.60 |
| $Na_2SO_4$ | 0.75 | 0.60 |
| $MgCl_2$ | 1.00 | 0.50 |
| $NH_4Cl$ | 1.00 | 0.50 |
| $NaCl$ | 0.75 | 0.50 |
| $CaCl_2$ | 0.75 | 0.50 |
| $NaNO_3$ | 0.20 | 0.15 |
| $KNO_3$ | 0.15 | 0.10 |
| $Ca(NO_3)_2$ | 0.20 | 0.10 |
| $KH_2PO_4$ | 0.50 | 0.30 |

the sulfates and the chlorides, but to a much smaller extent than the nitrate. The injurious action of the nitrate is exerted both on the growth of the organism, as indicated by the smaller amount of carbon assimilated in proportion to the sulfur oxidized, and on the respiration of the organism, as will be shown later.

*Concentration of Phosphates and Sulfur Oxidation.*—In the study of sulfur oxidation, a buffer has to be employed in the medium, otherwise the reaction will rapidly become acid. The phosphates form an excellent buffering agent for the study of the activities of this organism, particularly because they are of inorganic nature and can be adjusted on the acid side of neutrality. The concentration of

phosphates on the sulfur oxidation has also an important practical application in the composting of sulfur, rock phosphate, and soil, whereby the sulfuric acid formed from the oxidation of sulfur by the organism acts upon the rock (tri-calcium) phosphate converting it into mono-calcium phosphate. The influence of phosphates is found to become injurious in concentrations of 0.3 M, as shown in Fig. 3.

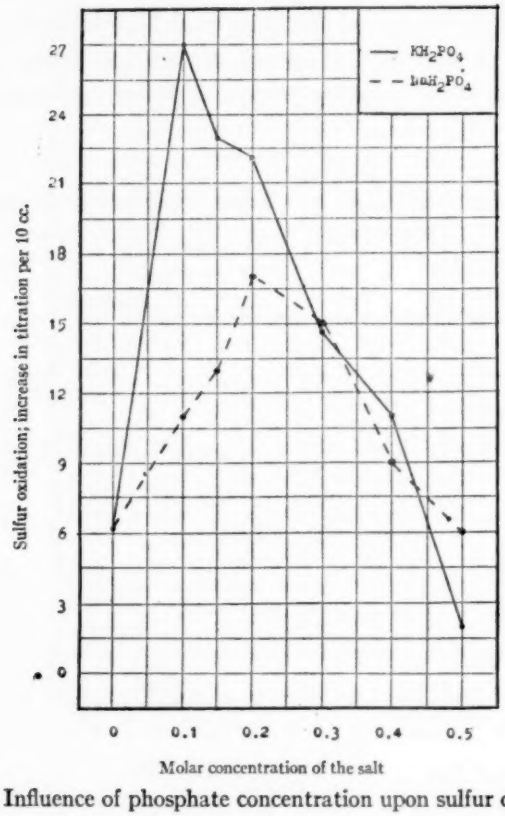


FIG. 3. Influence of phosphate concentration upon sulfur oxidation.

It is interesting to note that there is (Table VII) a gradual acclimatization of the organism to the concentration of the salt, with a prolonged period of incubation.

*Influence of Sulfur Concentration upon Sulfur Oxidation.*—With most cells it is very difficult to study the relation between concentration of nutrient and velocity of transformation due to the fact that the cells may be able to use reserve substances, or due to the difficult

differentiation between the amount of nutrient used for energy and that used for structural metabolism. The simpler the chemical composition of the nutrient the easier is the subject for investigation, particularly in the case of organisms where structural metabolism is distinct from energy utilization and where there are none or mere traces of the reserve materials, as in the case of nitrifying and sulfur-oxidizing bacteria.

Various amounts of sulfur were added to 100 cc. portions of medium, then sterilized as usual. At the end of 10 days incubation, the

TABLE VII.

*Influence of Concentration of Phosphates on the Oxidation of Sulfur by Sulfomonas thiooxidans.*

| Molar concentration. | $KH_2PO_4$ | | | | $NaH_2PO_4$ | | | |
|---|---|---|---|---|---|---|---|---|
| | 6 days. | 9 days. | 15 days. | | 6 days. | 9 days. | 15 days. | |
| | pH* | pH | pH | Titer.† | pH | pH | pH | Titer. |
| None. | 1.2 | 1.2– | 1.2– | 0.62 | | | | |
| 0.10 | 2.4 | 1.55 | 1.2 | 2.7 | 2.4 | 2.0 | 1.7 | 1.1 |
| 0.15 | 3.0 | 2.0 | 1.3 | 2.3 | 2.4 | 1.9 | 1.7 | 1.3 |
| 0.20 | 3.0 | 2.0 | 1.4 | 2.2 | 2.5 | 1.9 | 1.65 | 1.7 |
| 0.30 | 3.3 | 2.45 | 2.0 | 1.4 | 2.9 | 2.25 | 1.9 | 1.5 |
| 0.40 | 3.9 | 3.2 | 2.4 | 1.1 | 3.9 | 3.6 | 2.4 | 0.9 |
| 0.50 | 4.2 | 4.2 | 3.6 | 0.2 | 4.2 | 3.4 | 2.6 | 0.6 |

* pH of control media is about 4.2.

† Titer = increase in titratable acidity over control, in terms of cc. 0.1 N NaOH for 1 cc. of culture.

amount of sulfur oxidized was determined and the results are presented in Table VIII and Fig. 4.

The curve (Fig. 4) obtained for the correlation between sulfur concentration and oxidation velocity is exactly the same as that found by Harden, for the correlation between sugar concentration and fermentation velocity by yeasts, and by Meyerhof, for the relation between nitrite concentration (in small amounts) and nitrate formation.

Meyerhof obtained the maximum velocity with 0.1 per cent $NaNO_2$, due to the fact that increasing concentrations of the nitrite become toxic to the organism. In the case of *Sulfomonas thiooxidans*, there is no injurious effect from the increase of the sulfur oxidation

TABLE VIII.

*Relation between Sulfur Concentration and Oxidation Velocity.*

| Amount of sulfur per 100 cc. of medium. | Sulfur oxidized to sulfate. | Increase in titer. |
|---|---|---|
| *gm.* | *mg. of S* | |
| 0.025 | 19.91 | 0.12 |
| 0.100 | 65.70 | 0.41 |
| 0.500 | 189.67 | 1.20 |
| 1.000 | 210.49 | 1.34 |
| 2.000 | 264.93 | 1.70 |
| 5.000 | 390.55 | 2.51 |
| 10.000 | 413.41 | 2.68 |
| 25.000 | 504.93 | 3.25 |
| 50.000 | 502.93 | 3.28 |

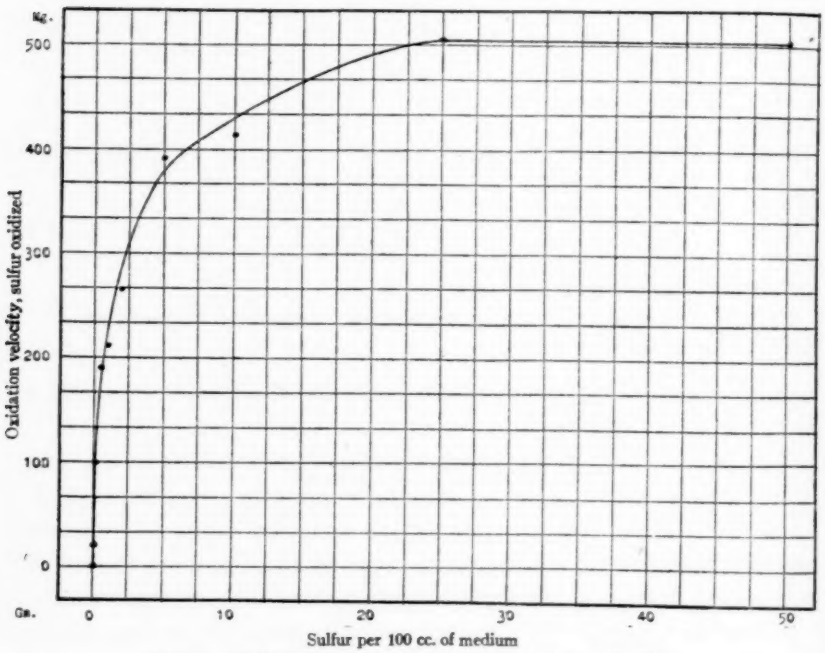


FIG. 4. Sulfur concentration and oxidation velocity.

even above the maximum which is about 25 per cent. It is important to note that the same interrelationship exists between the respiratory power of an organism and source of energy present in solution and that of an organism and source of energy existing in a solid phase. The very large surface exposed by the powdered sulfur (about 100 mesh) to the action of the organism seems to make the conditions similar.

TABLE IX.

*Influence of Dextrose and Peptone on Sulfur Oxidation by Sulfomonas thiooxidans.*

| Dextrose. | pH | Sulfate as S in 100 cc. |
|---|---|---|
| *per cent* | | |
| Control. | 4.0 | 11.20 |
| 0 | 1.4 | 176.22 |
| 0.02 | 1.4 | 170.97 |
| 0.20 | 1.4 | 164.40 |
| 1.00 | 1.4 | 194.54 |
| 5.00 | 1.4 | 179.20 |
| 10.00 | 2.8 | 24.00 |
| 20.00 | 3.8 | 12.30 |
| **Peptone.** | **pH** | **Sulfate as S in 100 cc.** |
| *per cent* | | |
| 0.01 | 1.4 | 188.70 |
| 0.1 | 2.0 | 83.60 |
| 1.0 | 2.8 | 19.40 |
| 2.5 | 4.0 | 11.20 |

*Influence of Dextrose and Peptone on Sulfur Oxidation.*—Winogradsky and Meyerhof pointed out that glucose will injure growth of nitrifying bacteria in 0.001 molar solution, but respiration is not affected by 0.2 molar concentration and is only injured 40 per cent in 0.6 molar (22 per cent). To amino compounds, respiration and growth are equally sensitive.

By adding various amounts of dextrose and peptone to 100 cc. quantities of media, sterilizing and inoculating in the regular manner, the following results were obtained after 7 and 14 days incubation, as shown in Table IX.

The organism is not injured in its activities by dextrose up to 1 per cent concentration. At 5 per cent, a decided injurious effect sets in. However, it takes nearly 20 per cent concentration of dextrose to stop growth and sulfur oxidation. Peptone injures the sulfur-oxidizing power of the organism even in small amounts. The uninjurious and even slightly stimulating effect of dextrose upon the respiration of the organism and the toxic effect of peptone and amino compounds has been pointed out elsewhere (Waksman and Starkey).

TABLE X.

*Influence of Dextrose on the Carbon Assimilation by Sulfomonas thiooxidans.*

| | Dextrose. | | |
|---|---|---|---|
| | Control. | Inoculated. | |
| | | Flask 1. | Flask 2. |
| C in 100 cc., *mg*. | 22.94 | 25.61 | 29.45 |
| | 24.60 | 25.77 | 28.32 |
| Average | 23.77 | 25.69 | 28.88 |
| Increase over control | | 1.92 | 5.11 |
| Sulfate as S in 100 cc., *mg*. | 10.26 | 87.93 | 200.79 |
| | 9.88 | 85.51 | 203.64 |
| Average | 10.07 | 86.72 | 202.22 |
| Increase over control | | 76.65 | 192.15 |
| $\frac{S}{C}$ | | 39.9 | 37.6 |
| pH | 3.6 | 1.7 | 1.2 |
| Titer | 0.16 | 0.67 | 1.41 |

To learn whether the presence of dextrose in the medium leads to a diminution in the carbon assimilation from carbon dioxide in the atmosphere, the organism was grown in the presence of small amounts (0.066 per cent) of dextrose for 10 and 16 days. The preparation of culture and cultivation of organism were followed by the procedure previously outlined. The results are given in Table X.

The S:C ratio, in the presence of dextrose, is thus found to be 38.8, or somewhat higher than the normal ratio, which is only 31.82. This

is not due to the assimilation of carbon from the dextrose, but to a natural variability of the growth of the organism or to a disturbed change in the metabolism of the organism. Any substance, whether of an organic or an inorganic nature, stimulating or injuring growth, effects the sulfur-carbon ratio, as will be further shown in the case of the nitrate. We can thus again confirm the fact that dextrose is not used either as a source of carbon or as a source of energy by the organism. This is true also of other carbohydrates and proteins, as found in the case of peptones.

*Influence of Nitrates on Sulfur Oxidation.*—It has been found in various studies carried out in this laboratory[2] that nitrates exert a depressive action upon sulfur oxidation, both in soil and in solution. As shown in Table VI, a concentration of 0.15 to 0.20 molar of the various nitrates is sufficient to repress completely the growth of *Sulfomonas thiooxidans*. This depressive action of the nitrate is the same when ammonium sulfate is present in the medium, indicating that the nitrate is directly toxic to the organism and not merely that it is an unfavorable nitrogen source. This action is exerted both on the growth and the respiration of the organism. By determining the sulfur-carbon ratio in the presence of nitrate, it is found to be more than twice as large as the normal ratio, indicating a distinct injurious effect upon the amount of carbon assimilated for a certain quantity of sulfur oxidized. By adding the nitrates to a fully grown culture and determining their influence on the respiration of the organism, by a method described elsewhere (Waksman and Starkey), it was found that 0.025 molar concentration of the various nitrates will repress respiration to about 25 per cent of normal, 0.05 molar to about 10 per cent, and 0.1 molar will repress respiration completely.

*Influence of Reaction upon the Activities of the Sulfur-Oxidizing Organism.*—Attention has been called to the fact elsewhere that the optimum reaction for the activities of *Sulfomonas thiooxidans* is at pH of 2.0 to 4.0 with limiting reactions at 6.5 and 0.6. However, this is true of the culture kept on artificial culture media, of a distinctly acid reaction, for a number of generations.

When freshly isolated, particularly from a neutral or slightly alkaline soil where sulfur oxidation has taken place, it may grow readily in

[2] Particularly in the work of Allison (to be published).

neutral or slightly alkaline reactions, although when isolated from acid soils, it seems to have as narrow a range of reaction.

The question of influence of reaction, as expressed in terms of hydrogen ion concentration as well as total concentration of acid, was studied both by adding different quantities of sulfuric acid to the regular medium (Table XI, Fig. 5) and by making up a series of media of various buffer contents. Molar concentrations of $H_3PO_4$, $KH_2PO_4$,

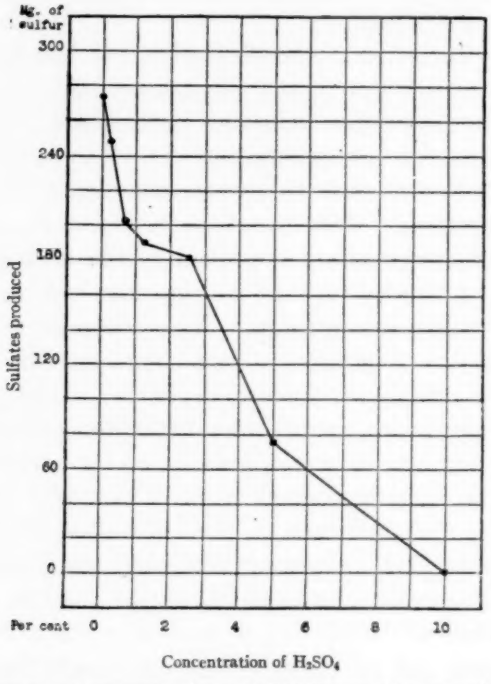


FIG. 5. Influence of concentration of $H_2SO_4$ on the growth of *Sulfomonas thiooxidans*.

$K_2HPO_4$, and NaOH were used, and 25 cc. of the proper buffer mixture were added to 475 cc. of the regular medium (free from phosphate) to give the range of reactions (Fig. 6).

The large concentrations of sulfuric acid that the organism can stand without injury are of interest. The optimum growth takes place when no acid is originally added to the medium (Table XI). This is in accord with the law of mass action. The organism still

grows when the medium contains 0.5 molar concentration of sulfuric acid, equivalent to nearly 5 per cent concentration of the acid. The growth in 0.25 molar concentration is almost near the optimum and, in many instances, no better sulfur oxidation is obtained even in normal media. The concentrations of sulfuric acid that the or-

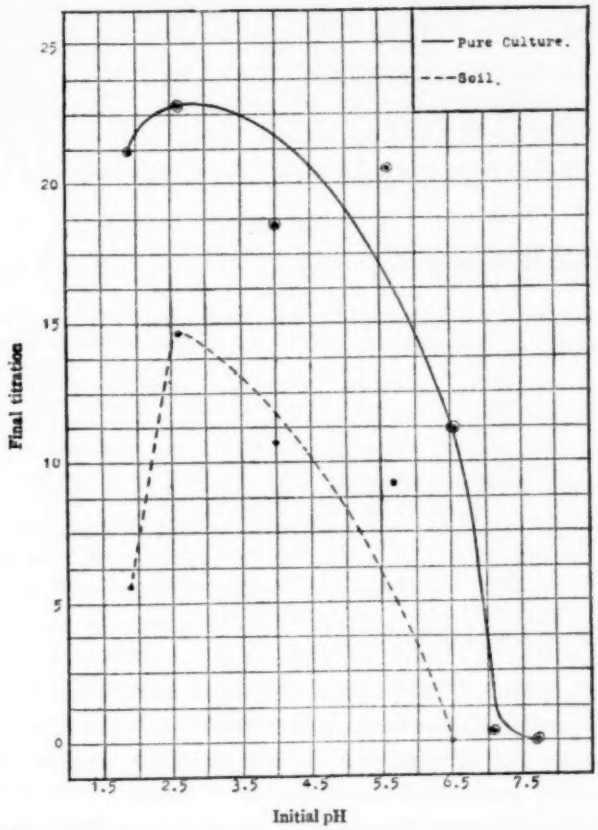


FIG. 6. Correlation between the hydrogen ion concentration of the medium and the growth of *Sulfomonas thiooxidans*.

ganism can stand without being destroyed are above 0.5 molar. In some cases, particularly when 10 to 25 parts of sulfur are added to 100 cc. of medium, sufficient acid is produced from the oxidation of sulfur to be equivalent to 1.5 normal alkali; the organism remains alive and capable of further oxidation even in these acid concentrations.

*Growth and Respiration.*—It was pointed out elsewhere (Waksman and Joffe (1922, *a*)) that the course of sulfur oxidation by *Sulfomonas thiooxidans* obeys the law of autocatalysis. When a culture is taken at the maximum period of its growth, and filtered free from sulfur, it can be used very readily for studies of respiration, in a manner similar to those carried out by Meyerhof for nitrifying bacteria. This method is usually carried out as follows: The organism is grown for 3 to 5 days, on the regular medium, in large 1 liter flasks, till a prominent turbidity is produced indicating active development. The culture is then filtered through paper free from sulfur. 50 cc. portions of the culture are then added to 1 gm. portions of sulfur in small flasks and incubated

TABLE XI.

*Influence of Concentration of $H_2SO_4$ upon the Oxidation of Sulfur by Sulfomonas thiooxidans.**

| Approximate molar concentration of $H_2SO_4$. | Titer per 1 cc. 0.1 N alkali. | | | Sulfate oxidized as sulfur in 100 cc. | | |
|---|---|---|---|---|---|---|
| | Control. | Inoculated. | Increase. | Control. | Inoculated. | Increase. |
| 0 | 0.58 | 2.70 | 2.12 | 13.2 | 288.2 | 275.0 |
| 0.025 | 1.07 | 2.89 | 1.82 | 100.3 | 349.6 | 249.3 |
| 0.062 | 1.80 | 3.29 | 1.49 | 242.7 | 443.9 | 201.2 |
| 0.125 | 3.03 | 4.45 | 1.42 | 474.8 | 665.0 | 190.2 |
| 0.250 | 5.89 | 6.85 | 0.96 | 844.3 | 1,026.8 | 182.5 |
| 0.500 | 10.18 | 10.75 | 0.57 | 1,559.8 | 1,635.3 | 75.5 |
| 1.000 | 20.03 | 19.98 | −0.05 | 3,134.3 | 3,075.0 | −59.3 |

* Period of incubation 12 days.

for 12 to 24 hours. The difference in titration or sulfate content is a reliable index of the amount of sulfur oxidized in this period of time. By adding to the culture various stimulants or depressive agents, we can readily study their influence upon the respiration of the organism as expressed in the amount of sulfur oxidized in the brief period of time. We can also study by this method the influence of gas pressure upon respiration as well as the influence of other treatments which in any way modify respiration. The results obtained by this method indicate that by increasing the $CO_2$ pressure in the atmosphere, respiration is greatly stimulated, while reducing the $CO_2$ pressure or using an inert atmosphere, respiration is largely repressed.

SUMMARY.

1. It is shown that *Sulfomonas thiooxidans* oxidizes elementary sulfur completely to sulfuric acid. Sodium thiosulfate is oxidized by this organism completely to sulfate. *Sulfomonas thiooxidans* differs, in this respect, from various other sulfur-oxidizing bacilli which either produce elementary sulfur, from the thiosulfate, or convert it into sulfates and persulfates.

2. The organism derives its carbon from the $CO_2$ of the atmosphere, but is incapable of deriving the carbon from carbonates or organic matter.

3. The S:C, or ratio between the amount of sulfur oxidized to sulfate and amount of carbon assimilated chemosynthetically from the $CO_2$ of the atmosphere, is, with elementary sulfur as a source of energy, 31.8, and with thiosulfate 64.2. The higher ratio in the case of the thiosulfate is due to the smaller amount of energy liberated in the oxidation of sulfur compound than in the elementary form.

4. Of the total energy made available in the oxidation of the sulfur to sulfuric acid, only 6.65 per cent is used by the organism for the reduction of atmospheric $CO_2$ and assimilation of carbon.

5. Sulfates do not exert any injurious effect upon sulfur oxidation by *Sulfomonas thiooxidans*. Any effect obtained is due to the cation rather than the sulfate radical. Nitrates exert a distinctly injurious action both on the growth and respiration of the organism.

6. There is a definite correlation between the amount of sulfur present and velocity of oxidation, very similar to that found in the growth of yeasts and nitrifying bacteria. Oxidation reaches a maximum with about 25 gm. of sulfur added to 100 cc. of medium. However, larger amounts of sulfur have no injurious effect.

7. Dextrose does not exert any appreciable injurious effect in concentrations less than 5 per cent. The injurious effect of peptone sets in at 0.1 per cent concentration and brings sulfur oxidation almost to a standstill in 1 per cent concentration. Dextrose does not exert any appreciable influence upon sulfur oxidation and carbon assimilation from the carbon dioxide of the atmosphere.

8. *Sulfomonas thiooxidans* can withstand large concentrations of sulfuric acid. The oxidation of sulfur is affected only to a small

extent even by 0.25 molar initial concentration of the acid. In 0.5 molar solutions, the injurious effect becomes marked. The organism may produce as much as 1.5 molar acid, without being destroyed.

9. Growth is at an optimum at a hydrogen ion concentration equivalent to pH 2.0 to 5.5, dropping down rapidly on the alkaline side, but not to such an extent on the acid, particularly when a pure culture is employed.

10. Respiration of the sulfur-oxidizing bacteria can be studied by using the filtrate of a vigorously growing culture, to which a definite amount of sulfur is added, and incubating for 12 to 24 hours.

The authors wish to express their thanks to Miss C. Wark for her faithful assistance in the experimental work.

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# THE PHAGOCYTOSIS OF SOLID PARTICLES.

## IV. Carbon and Quartz in Solutions of Varying Acidity.

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In the preceding paper of this series (1) it was shown that carbon particles are ingested by rat leucocytes three or four times as readily as quartz particles of approximately the same size. Variations were found, however, in this ratio between the rates of ingestion of carbon and quartz during the course of a single experiment which suggested that significant variations might be produced by varying the fluid environment of the cells. The present paper includes experiments which indicate a reversal of this ratio with acidification of the solution, quartz becoming more readily ingested than carbon. It was hoped that a further study of this comparatively simple phagocytic system of leucocytes with quartz and carbon particles might yield results which could be interpreted in terms of physical chemistry and hence give some quantitative experimental clue as to the fundamental nature of phagocytosis.

### *a. The Carbon-Quartz Ratio at Varying pH.*

The method used for these experiments was the "film method" previously (1) described. Briefly, it consists in mixing together suspensions of leucocytes obtained from a peritoneal exudate in the rat, quartz and carbon particles of uniform sizes, serum, and phosphate mixtures for the control of the hydrogen ion concentration. This mixture is allowed to run under a cover-glass supported on the slide by cover-glass fragments, which is then sealed with paraffin to prevent drying. A count is immediately made of the relative numbers of particles of the two sorts originally present. The preparation is then placed on the warm stage and, as the leucocytes creep about on the slide ingesting the particles, counts are made of the numbers of quartz

and carbon particles which appear inside the cells. Later in the experiment, when phagocytosis becomes more complete, a better measure of the relative rates of phagocytosis is obtained by counting the numbers of particles still free; *i.e.*, neither inside nor stuck to the cells.

TABLE I.

*Increase of the Ratio of Ingested Carbon to Ingested Quartz Particles with Increase in pH.*

| Experiment No. | Serum. | pH | Per cent carbon / Per cent quartz | Experiment No. | Serum. | pH | Per cent carbon / Per cent quartz |
|---|---|---|---|---|---|---|---|
| | *per cent* | | | | *per cent* | | |
| 1 | 17 | 7.2 | 0.88 | 6a | 10 | 7.1 | 2.28 |
| | | 7.6 | 1.8 | | | 7.3 | 2.6 |
| | | | | 6b | 10 | 7.1 | 0.87 |
| 2 | 7 | 6.4 | 0.43 | | | 7.3 | 4.0 |
| | | 7.3 | 1.39 | | | | |
| | | 7.6 | 2.93 | 7 | 10 | 6.6 | 0.58 |
| 3 | 10 | 6.5 | 0.84 | | | 7.0 | 0.73 |
| | | 7.0 | 2.03 | | | 7.2 | 1.5 |
| | | 8.0 | 1.49 | | | 7.4 | 1.8 |
| 4 | 10 | 7.2 | 1.32 | 8a | 36 | 6.6 | 1.3 |
| | | 7.5 | 1.56 | | | 7.3 | 2.2 |
| 5 | 17 | 7.0 | 1.21 | 8b | 36 | 6.6 | 0.8 |
| | | 7.2 | 1.90 | | | 7.3 | 2.1 |

pH was measured electrometrically. Ratios were calculated from counts of particles inside the cells except in Experiments 6b and 8b where the particles outside the cells were counted and those inside calculated by difference. pH was controlled by phosphate buffers except in Experiment 8a where the acid reaction was obtained by equilibrating with 4 per cent $CO_2$ in room air (see text).

The results of a series of eight experiments with this method are collected in Table I. Each experiment involves so much counting that it is difficult to compare more than two or three solutions at once. The pH was measured electrometrically in most cases, but in a few it was estimated accurately enough from that of the phosphate mixtures used. In this table all the ratios are based on the percentages of particles found inside the cells except as noted. In a number of other experiments the ratio between the numbers of quartz and carbon

particles free at the close of the experiment was determined, and it was always found that there was relatively more carbon free in acid solutions and relatively more quartz free in alkaline solutions. This confirms the results in Table I which show that the percentage of carbon particles inside the cells increases relatively to the quartz with increasing alkalinity. If the pH at which the ratio equals 1 is determined by interpolation from the results in Table I, the points indicated are pH 6.93, 7.17, 7.07, 7.25, 7.11, and 6.71, the average being 7.04. These figures are, of course, far from accurate, but the agreement among them is good enough to warrant the general conclusion that *quartz is ingested more readily than carbon in acid solutions, but carbon is ingested more readily than quartz in alkaline solutions.* It should be particularly emphasized that this conclusion does not contradict the fact that the optimum pH for phagocytosis of both carbon and quartz particles by this method is at or near neutrality. This optimum is best interpreted as due to the physiological effect of the pH on the leucocytes rather than on the particles.

Special mention should be made of Experiment 8a in Table I where the acid reaction was obtained by equilibrating with room air containing 4 per cent $CO_2$, the resulting pH being measured electrometrically using 4 per cent $CO_2$ in hydrogen for the gas. The method of performing this experiment is simple in principle but rather difficult technically.

A cover-slip was supported as usual on a slide and under its opposite edges two fine capillary tubes were inserted. One of these was drawn from the bottom of a small 1 cc. test-tube in which the mixture of cells and particles was placed. The edges of the cover-slip were then sealed with paraffin. Gas was then introduced through one capillary tube passing under the cover-slip and escaping by bubbling up through the suspensions in the test-tube. This was continued until the system was in equilibrium. The particles were not added until just before turning off the gas in order to minimize the clumping which otherwise occurs. Finally the gas is shut off, a little of the mixture is sucked quickly back under the cover-slip, the capillary tubes are broken off, and the ends sealed over with paraffin.

One point, possibly of some theoretical significance, and which came to light in these experiments, is the fact that when carbon was prepared with the use of 0.4 per cent acacia as a stabilizer, the addition of acid failed to prevent the carbon from being taken up better than the quartz. Acacia also affects the surface of the carbon in such a way

that the negative charge on the particle cannot be reversed by the addition of acid. The ratios observed (always > 1) between the numbers of carbon and quartz particles ingested were 2.4, 2.45, and 2.2 at pH 6.0; 1.5, 1.46 at pH 6.2; 1.57 and 1.56 at pH 6.7; and 3.5 at pH 7.6. Figures in Table I, it will be remembered, showed ratios as low as 0.5 in acid solutions, indicating that the carbon was taken up only one-half as well as the quartz.

*b. The Phagocytosis of Manganese Dioxide and Manganese Silicate.*

On account of experiments from this laboratory by Drinker and Shaw (2) on the distribution of manganese dioxide and manganese

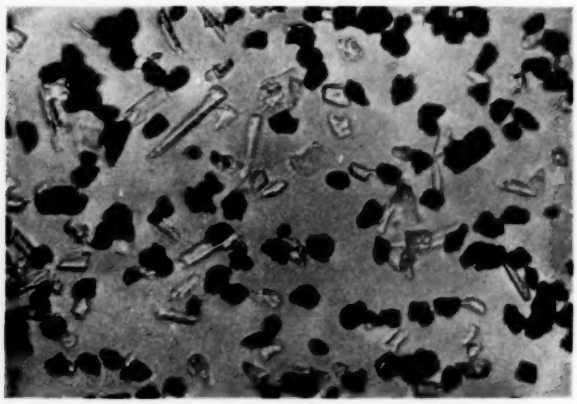

FIG. 1. Photograph of a mixture of the suspensions of manganese dioxide and manganese silicate used in the phagocytosis experiments. Leucocytes are attracted toward the black $MnO_2$ particles which they ingest leaving the silicate. The average diameter of the particles was somewhere between 3 and 5 microns.

silicate after intravenous administration in the cat and the relative rates of their removal from the blood stream, it became important to determine the relative rates of phagocytosis of these two kinds of particles. For this purpose uniform suspensions were prepared by fractional centrifugation in 0.4 per cent acacia. The acacia prevents the manganese dioxide from clumping and is indispensable. A photograph of the mixture of the two products is shown in Fig. 1. The diameters were not measured but were between 3 and 5 microns. The method used was the same as that described for carbon and

quartz. *The rate of ingestion of $MnO_2$ was astonishingly rapid.* In the first experiment the number of free $MnO_2$ particles fell from 105 to 22 per unit area in 12 minutes, during which time the silicate count over the same area had not perceptibly changed from 106. In the second experiment 78 $MnO_2$ particles were ingested per unit area in 10 minutes while 31 silicate particles were being ingested, approximately equal numbers of each being present originally. In a third experiment the free particles of $MnO_2$ decreased from 93 to 7 in 8 minutes while the silicate count decreased only from 99 to 67. In another experiment the rate of ingestion of quartz was compared with that of $MnO_2$ at pH 6.3, 7.0, and 7.6. The results are summarized in Table II. A change in pH from 6.3 to 7.6 causes therefore no reversal in the relative rates of phagocytosis of these two kinds of

TABLE II.

*Phagocytosis of Manganese Dioxide and Quartz with Varying pH.*

| pH | No. of particles per unit area at start. | | No. of particles still free after 20 min. | | No. of particles ingested after 10 min. | |
|---|---|---|---|---|---|---|
| | Quartz. | $MnO_2$ | Quartz. | $MnO_2$ | Quartz. | $MnO_2$ |
| 6.3 | 80 | 60 | 54 | 4 | 4 | 65 |
| 7.0 | 76 | 53 | 59 | 8 | 7 | 86 |
| 7.6 | 69 | 47 | 67 | 9 | 15 | 62 |

particles, like that found for carbon and quartz. The ingestion of the manganese dioxide, however, was so rapid that there was not sufficient time to make the counts accurate enough to exclude the possibility of some effect.

### *c. Are Meetings between Cells and Particles Fortuitous?*

It has been shown by Commandon (3) by means of moving pictures that leucocytes are attracted by starch grains. It was of interest therefore to determine whether the preference shown for carbon and for manganese dioxide exhibited by leucocytes is due to a greater ease of ingestion or to an attraction between them resulting in more frequent meetings.

For this purpose a preparation was made under the cover-slip as for the "film method." One field was chosen which was divided into

squares by a suitably ruled ocular disc. While the cells were still inactive, having just come from the cold room, the positions of each carbon and each quartz particle in the field were plotted on a duplicate chart in the note-book. The heating current was then passed through the warm stage and as ingestion began each square was rapidly inspected in turn. As soon as any particle was seen in contact with a leucocyte a circle was drawn around it in the note-book and the time noted. Later collisions with this same particle were disregarded. It made no difference in the result, therefore, whether the particle was ingested or whether the leucocyte crept away again and left it. Thus the experiment closely resembled a game of tag in which the leucocytes were "it," the particles being out of the game when tagged. By working rapidly it was just possible to keep a fairly accurate record of 30 to 40 particles of each kind but there was considerable room for errors of judgment. The method was nevertheless more than accurate enough to detect the large difference between $MnO_2$ and quartz.

Seven experiments of this sort have been tried with carbon and quartz particles. The results are difficult to tabulate briefly without exaggerating their significance, but they can be quite accurately summarized by two perfectly representative examples. In one experiment a field was selected for observation containing 44 carbon and 38 quartz particles and 77 leucocytes. After 24 minutes on the warm stage there were 1 quartz particle and 12 carbon particles to be seen inside the leucocytes. Yet the number of observed collisions between leucocytes and quartz had been 36 as compared to 37 between the leucocytes and the carbon. In another experiment a field of 38 carbon and 42 quartz particles showed after 28 minutes only 4 quartz to 20 carbon particles ingested, while 34 quartz and 33 carbon particles had been seen at one time or another in contact with the leucocytes. By careful observation of such experiments leucocytes can frequently be seen in contact simultaneously with both quartz and carbon particles, finally ingesting, however, only the carbon. Likewise in other experiments where the acidity is such that quartz particles are more readily ingested, there is ample evidence to show that cells are unable to keep hold of the carbon particles although the quartz is tenaciously retained. In no cases, however, did the rates of collision

of leucocytes with the two kinds of particles show variations which could not be attributed to unwitting errors of judgment or similar causes.

In some earlier experiments it was found that carbon particles which had been centrifuged out of 0.4 per cent acacia solution and then resuspended in distilled water before mixing with the leucocytes were ingested much more rapidly than untreated carbon. The cause of this result has not been ascertained but it suggested the possibility that leucocytes might be attracted toward carbon so treated but not towards normal carbon. Four experiments were therefore tried using carbon which had been centrifuged from 0.4 per cent acacia in which they had been kept (unsterile) for $2\frac{1}{2}$ years. It was estimated that the

TABLE III.

*Rate of Collision of Leucocytes with Manganese Dioxide and Manganese Silicate.*

| Experiment No. | No. of collisions per min. | |
|---|---|---|
| | $MnO_2$ | $MnSiO_3$ |
| 1 | 3.3 | 0.73 |
| 2 | 2.5 | 0.85 |
| 3 | 4.0 | 1.8 |
| 4 | 2.9 | 1.3 |
| 5 | 2.0 | 1.1 |
| Average..... | 2.9 | 1.2 |

carbon was ingested 1.8 times as readily as the quartz but the rates of collision, though slightly in favor of the carbon (1 to 1.2 times as fast as the quartz), could not be regarded as significant.

From these experiments there is reason to believe that *the encounters of cells with quartz and carbon particles are purely fortuitous.* In this connection it should be mentioned that Schaeffer (4) asserts that amebæ are attracted toward particles of glass.

The rates of collison of leucocytes with $MnO_2$ and quartz particles were also investigated by this same method. Five such experiments are readily summarized in Table III, in which the average number of collisions per minute is recorded. *The rate of collision of the leucocytes with the manganese is seen to be* $\frac{2.9}{1.2} = 2.4$ *times as great as with the*

*quartz.* This difference indeed was almost obvious from mere inspection. Pseudopods often seemed to be put out in the direction of the manganese particles and in one case, recorded in Fig. 2, a leucocyte seemed obviously to be attracted from a distance of perhaps 30 microns toward a group of two manganese and one quartz particle. From its unusually rapid movements the leucocyte appeared to be "in haste" and its path was directly blocked by the quartz particle. Two pseudopods were put out "straddling" this obstruction, each of which promptly ingested a manganese particle. After this feat the leucocyte rounded up and wandered away with its twin load leaving the quartz particle undisturbed. The whole performance could not have lasted over 2 minutes. Similar but less striking occurrences

FIG. 2. Diagram of an actual case in which a leucocyte was apparently attracted toward two particles of manganese dioxide which it ingested with such precision that it seemed almost "purposeful," an intervening particle of quartz being completely neglected. Time about 2 minutes.

were frequently observed. The acacia used in preparing the manganese could hardly have been responsible for this attraction since it was in large measure removed by 2 or 3 washings on the centrifuge before using the suspension. Moreover, carbon particles similarly treated showed no comparable attraction for leucocytes. It seems probable, therefore, that the attraction of the $MnO_2$ is due to its slight solubility which is particularly noticeable in the presence of carbonic acid and organic matter (Drinker and Shaw (2)).

It has been mentioned in a foot-note in a previous paper (1) that when a mixture of manganese silicate and manganese dioxide particles is shaken up with chloroform or oil the particles are found collecting in the interfaces between the drops of chloroform or oil and the water. If counts are made of the numbers of the two kinds of particles still

free after unit additions of chloroform or oil, it is found that the silicate particles collect in the interfaces more rapidly than the manganese dioxide, leaving an increasing proportion of the latter in solution. Thus it may be concluded that artificial leucocytes of chloroform or oil ingest the silicate more rapidly than the dioxide, the result being thus exactly contrary to the findings with live material.

### *d. Penicillium Spores versus Quartz.*

One experiment was tried with *Penicillium* spores and quartz particles. Only single spores and double spores were counted, longer chains being totally disregarded. In spite of the fact that the quartz particles were somewhat larger than the spores, the latter were carefully picked out by the leucocytes so that after 2 hours 58 per cent of the spores and only 9 per cent of the quartz had been ingested. The corresponding ingestion after 4 hours was 65 per cent for the spores and 25 per cent for the quartz.

### *e. Relative Ingestibility of Large and Small Particles.*

In the course of some experiments on the relative rates of ingestion of various kinds of lead dusts[1] it became necessary to use some of the dusts in very small particles, and the question arose whether such extreme variations in the size would affect the rate of ingestion. It had already been ascertained (5) that a variation from 2 to 5 microns in diameter had no measurable influence on the rate of phagocytosis except in so far as the chances of collision were increased by increase in the size of the particle. This chance of collision could easily be calculated, it being equal to the sum of the diameters of the leucocyte and particle, provided all movement of the leucocytes was confined to two dimensions, as on a microscope slide. It was expected that the large particles would be taken up slightly less rapidly than would be predicted from the chances of collision alone because of the greater mechanical deformation of the cell necessary for their ingestion. It was a surprise to find that the small particles, approximately 0.9 micron in diameter were apparently ingested with considerable difficulty. This was determined by comparing the rates of ingestion of four sizes

[1] These were undertaken with the cooperation and at the suggestion of Dr. Alice Hamilton, and with the assistance of Mr. Jacob Fine.

of quartz particles of diameters 0.9, 2.8, 4.0, and 7.8 microns respectively. The results are shown in Table IV where the "expected" and "observed" ratios of the rates of phagocytosis of the small to large particles are compared. The figures show a fairly definite tendency for the smaller particles to be taken up less rapidly than the calculations predicted, especially where the 0.9 micron particles are concerned. In other cases (7.8 *versus* 2.8 microns) it is in fact doubtful

TABLE IV.

*Ratios of Ingestion Rates of Large and Small Quartz Particles.*

| Diameters of particles. | Expected ratio. | Observed ratio. |
|---|---|---|
| *microns* | | |
| 2.8 *vs.* 0.9 | 0.85 | 0–0.19 |
| | 0.85 | 0.35–0.54 |
| 7.8 *vs.* 0.9 | 0.61 | 0.06–0.28 |
| | 0.61 | 0.22–0.66 |
| 7.8 *vs.* 2.8 | 0.72 | 0.72 |
| | 0.72 | 0.48–0.63 |
| 4.0–0.9 | 0.7–0.8 | 0.32 |
| | | 0.1 –0.22 |
| | | 0.55–0.66 |
| | | 0.35 |
| | | 0.29–0.45 |

Expected ratios are the ratios of the chances of collision $\left(\frac{\text{small}}{\text{large}}\right)$ calculated from the diameters of the particles and leucocytes. Observed ratios are the ratios of the percentages of small to large particles ingested in equal times. Each line in the table represents a separate experiment. Where the observed ratios are smaller than the expected, the smaller particles are taken up less readily than expected.

whether the differences between expected and observed ratios are greater than the experimental error. There is no doubt, however, that under the conditions of these experiments *some factor other than surface tension* becomes significant in the phagocytosis of the 0.9 micron particles. In some experiments almost all the larger particles were found inside the cells before the ingestion of these smallest particles became greater than the experimental error of the counts.

Three possible explanations of this result are: (1) The small particles are excreted as rapidly as they are ingested. This obviously could not be true if phagocytosis were merely a matter of surface tension. (2) The small particles, being almost at the limit of microscopic visibility and in brownian movement, may be merely pushed aside by the leucocyte which may be regarded as experiencing the same difficulty which a child finds in "bobbing for apples" in a tub of water. (3) Small particles may give the cell less of a "stimulus" than the large particles, the response being correspondingly less.

It is regretted that time has not permitted a more thorough investigation of the rates of ingestion of different sizes of particles. These experiments, though preliminary in nature, are reported because they illustrate one definite quantitative method of testing the surface tension hypothesis. By this test it is found incomplete.

## DISCUSSION OF RESULTS.

What seems to be the most interesting and significant fact which has come out of these experiments on the phagocytosis of carbon and quartz particles is the reversal of preference at neutrality, carbon being taken better than quartz in alkaline solutions, quartz better than carbon in acid solutions. It would seem as if this fact might provide a means of discovering just what property of a particle it is which favors its ingestion by a leucocyte. Obviously the limiting factor in these experiments is something which concerns the surface of the solid particle. Either it may be related to the interface between the particle and the solution or to the interface (not necessarily surface tension) between the particle and the leucocyte. It cannot be due to the effect of a change in acidity on the leucocyte-solution interface, for the ingestion of the two different particles is differently affected by the same change.

To explain this fact a series of measurements on the particles themselves was undertaken. The most obvious property to investigate from this point of view was the electrical charge on the particle which is known to vary characteristically with the hydrogen ion concentration. On account of the ready agglutination of the carbon particles by acid it was thought easier to measure the charge by making membranes of the carbon and observing the electrical endosmosis through

the membrane. A rapid and simple method was devised which was accurate enough for this purpose.

A glass tube of about 6 mm. inside diameter open at both ends was fixed by means of a rubber stopper upright in a centrifuge tube and just off the bottom. A thick suspension of the particles to be investigated was then sedimented out in the centrifuge tube so that the open end of the small central tube was completely embedded in the sediment. A difference of potential of 50 to 90 volts was then established between the inside and the outside of the central tube by means of small platinum electrodes dipping in the supernatant liquid. The movement of the meniscus in the central tube was then observed before and after reversing the current and the average of the two readings was taken as a measure of the charge on the particle. A useful modification is to have the level of solution in the central tube higher than that outside. If the membrane leaks, the difference in the rates of filtration before and after reversal of the current gives a measure of the charge. In experiments lasting only 5 minutes there is no serious danger of products of electrolysis in the supernatant liquid affecting the charge on the particles embedded in the sediment. A more serious error, inherent in any membrane method, is the change of the fluid inside the membrane due to solubility of the particles or absorbed electrolytes.

By this method a considerable number of measurements were made of the charges on quartz and various types of carbon in solutions of varying pH. It is unnecessary to give these in detail since they add nothing essential to the observations of Gyemant (6) by a more difficult method which came to our attention afterwards. They showed, however, an isoelectric point for carbon at about $C_H$ $10^{-5}$ and a negative charge for quartz which diminished toward zero in normal or 0.1 normal HCl but never reached an isoelectric point. In the presence of acacia carbon behaved like quartz, there being no isoelectric point. Likewise it was found, as already stated, that in the presence of acacia quartz was never preferred to carbon even in acid solutions. It is probable, however, that no difference between the endosmotic charges of quartz and carbon existed in the actual phagocytosis experiments, for Davis has found in experiments from this laboratory (unpublished) that in the presence of minute amounts of serum, glass particles assume the isoelectric point of the serum proteins. This he established by the use of the method described above as well as by cataphoresis. If it is true that quartz becomes thus coated with the serum proteins, one would expect it to be even more true of carbon with its high adsorptive capacity. The measurements therefore make

it probable that both carbon and quartz have isoelectric points corresponding to those of the serum proteins, under the conditions of the phagocytosis experiments, and it becomes difficult to explain the observed differences in their behavior toward leucocytes by means of their endosmotic charges. Moreover, even if carbon and quartz did not have the same isoelectric points, one would expect that carbon would be taken up relatively better in acid solutions as it approaches its isoelectric point where the repulsion between it and the similarly charged leucocyte becomes zero, whereas experiment tells us that the reverse is true.

The charge on the manganese dioxide particles used for phagocytosis has been investigated by this same method and found to behave much like quartz having a negative charge in all solutions of HCl. Here again these endosmotic potentials offer no explanation of the differences detected by the leucocytes.

Freundlich (7) has pointed out in a recent series of papers that two different potentials can be measured across interfaces such as that between glass and water. These are (*a*) the endosmotic potential which he interprets as existing between the body of the solution and the fixed layer of liquid which remains adhering to the solid wall, and (*b*) the Nernst electrode potential which he believes to be the potential between the solid itself and the body of the solution. Whether this interpretation of the facts be valid or not, it seems not impossible that these electrode potentials might be concerned in phagocytosis instead of the endosmotic potentials. The impossibility of measuring the electrode potential of a leucocyte makes it difficult to test this hypothesis completely. Both carbon and glass, however, show increasing positive potentials with increasing hydrogen ion concentration so that there is no obvious qualitative difference between them which might explain the behavior of leucocytes in ingesting them.

A carbon electrode was prepared from the same material from which the suspensions were made by fixing the largest piece available, about the size of a pea, in a pair of tweezers. The whole electrode was then paraffined except for the tip of the carbon which was in contact with the solution, the tweezers being connected to the potentiometer. The potential of the carbon electrode was then measured against the saturated calomel electrode in solutions of varying pH.

Such carbon electrodes when first immersed in the solution have a potential of about 0.1 volt higher than the saturated calomel electrode (*i. e.* when measured

against an H electrode they give 0.1 volt higher P.D. than the saturated calomel electrode). In acid solutions this P.D. increases quite rapidly with time and after a day or two comes to approximate equilibrium at about 0.3 or 0.4 volt. In alkaline solutions the P. D. falls with time to 0 or −0.05 volt. Using buffered phosphate solutions between pH 6 and pH 10 it was found that there was no change with time in solutions between pH 8 and pH 9 approximately. Distilled water shaken up with carbon suspensions also gives pH readings between these limits. It would appear therefore that carbon is in equilibrium with slightly alkaline solutions. Pieces of a carbon rheostat used as electrodes gave confirmatory results.

Using Palmaer's (8) value of 0.5732 for the absolute potential of the 0.1 normal calomel electrode it may easily be calculated that the absolute potential of the carbon electrode at pH 5, *i.e.* at its isoelectric point, is between 0.6 and 0.7 volt. The difference between the endosmotic and the electrode potentials thus becomes evident.

The potential of the glass electrode was first measured by Haber and Klemensiewicz (9). Their findings were confirmed by Freundlich and more recently by Dr. W. T. Bovie and Mr. W. S. Hughes (unpublished), who have plotted the potential against the hydrogen ion concentration and find the charge increasingly positive with increasing acidity.

In conclusion it must be admitted that these experiments have not led to any explanation of the relative rates of ingestion of carbon and quartz particles by leucocytes which can be proved experimentally. The search for such an explanation was perhaps manifestly hopeless from the start in the present state of our knowledge of the physical chemistry of solid surfaces. Yet in comparison with any phagocytic system involving bacteria, where previous explanations have been attempted, this problem was extraordinarily simple. One great simplification is that there are two kinds of particles, each one serving as a control for the other and eliminating to a large extent the physiological variations in the leucocyte itself with change of pH. If an explanation could be found it seems certain that we should have made a substantial advance not only in the study of the mechanism of phagocytosis but in the study of the fundamental nature of living protoplasm. Progress in this direction is certainly to be made by the intensive study of comparatively simple cases such as that here discussed.

## SUMMARY.

1. Leucocytes ingest quartz particles more readily than carbon in acid solutions, and carbon more readily than quartz in alkaline solutions.

2. In the presence of acacia carbon is always preferred to quartz even in acid solutions.

3. Manganese dioxide particles are ingested by leucocytes with extraordinary rapidity as compared with manganese silicate or quartz.

4. Leucocytes are not attracted toward carbon or quartz particles but manganese dioxide exerts a distinct attraction for them.

5. Spores of *Penicillium* are ingested more readily than quartz.

6. Very small quartz particles, 1 micron in diameter, are not ingested as readily as larger particles of the same material. This result being contrary to the predictions of surface tension indicates that some other factor is involved in the ingestion of these small particles.

7. Measurements of the carbon electrode potentials and the cataphoretic charges on the particles have failed to supply an explanation for the varying relative rates of ingestion of carbon and quartz with varying hydrogen ion concentration.

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# MUSCLE TENSION AND REFLEXES IN THE EARTHWORM.

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## I.

It was first shown by Friedländer[1] that a decapitated piece of earthworm would perform normal peristalsis, with perfect coordination between the segments, if it were pulled forward over a rough surface. This experiment proved that the brain was not necessary to locomotion but did not clearly decide the question as to whether the impulse to movement was given by sensory stimulation of the integument in contact with the surface or by tension of the muscles themselves as the result of pulling.

The first possibility, namely, contact stimulation of the integument, has been shown to be sufficient cause to initiate peristalsis in a suspended preparation of the worm.[2] On the other hand, if sensory stimulation of the dermal layer could be avoided it would be possible to test the value of muscle tension alone as a cause of peristalsis. For this purpose it is only necessary to put the worm into an M/8 solution of $MgCl_2$, allowing it to remain until there is no response to the stroke of a moist camel's hair brush. If a piece of suitable length be cut from the median portion of the worm and arranged in the usual fashion for making a kymographic record, the effect of tension in initiating peristalsis can be demonstrated. It should be noted at the outset that no spontaneous contractions occur and that stroking the preparation with a brush does not initiate peristalsis. Next, let a weight be attached to the lower end of the piece so as to exert a tension of 1 or 2 gm. The extension resulting from the force of the weight is immediately followed by further active extension of the worm for a

[1] Friedländer, B., *Arch. ges. Physiol.*, 1894, lviii, 168.

[2] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915–16, xxxix, 140.

few millimeters, the result of contraction of the circular musculature.[3] This movement is succeeded by strong contraction of the longitudinal musculature (Fig. 1). Alternate extension and retraction of the preparation take place as long as the weight remains or until the muscles are fatigued. It should be noted that when tension is the

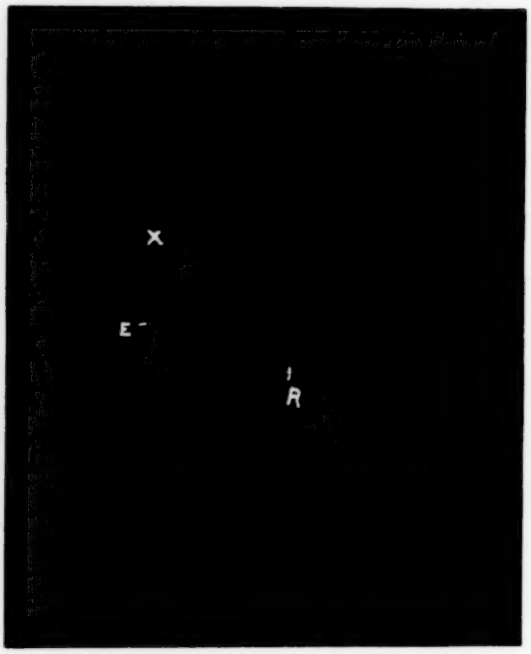


FIG. 1. The effect of tension in producing peristalsis in a piece of earthworm, the cutaneous sensory cells of which had been anesthetized by $MgCl_2$. *X* indicates the point at which the weight of 1 gm. was applied; *E* shows the beginning of the phase of active extension; *R* shows the point at which the weight was removed. Contraction is shown by upstroke; relaxation by downstroke.

exciting cause of peristalsis the first phase of the wave is extension; but if dermal stimulation is the exciting cause, extension is the first phase of peristalsis only in case stimulation is applied at the anterior end of the preparation. If the sensory stimulation be applied at the posterior end, the first phase of peristalsis is a shortening.[4]

[3] See also Biedermann, W., *Arch. ges. Physiol.*, 1904, cii, 487.

[4] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915, xxxix, 140.

It is evident that receptors for tension exist in the musculature of the worm, since, in the foregoing experiments cuticular sense cells have been eliminated by magnesium anesthesia. Tension on the nerve cord itself has no effect.[5] Since contraction of the circular muscles is the first response to passive extension, it is clear that the receptors which are stimulated by tension of the longitudinal musculature are connected by nervous paths with the circular muscles. Such an arrangement may well be the basis of the reciprocal innervation phenomena noted by Garrey and Moore[6] and by Knowlton and Moore.[7] The anatomical basis of this receptor system has been demonstrated by Dawson.[8] He has proven the existence of nerve cells of sensory type, imbedded for the most part in the ventral musculature of the earthworm. Presumably these are the neurons which act as receptors for the tension reflex just described. The results obtained by Straub,[9] Budington,[10] and Bovard[11] lend support to this hypothesis. Straub removed the ventral nerve cord from thirty segments of a worm and after 8 days tested the preparation for the tension reflex. He obtained positive results and supposed this to be the reaction of the muscle cells to their own stretching, since he afterward found no evidence of regenerated ventral ganglia. Nevertheless, Budington's work shows that the pieces of worm used by Straub must have contained some nervous tissue outside the region of the ventral cord, and that therefore Straub's results must have been the result of neuron reflexes. Budington found, on the contrary, that if the precaution be taken to remove the underlying strip of the myodermal wall with the nerve cord, it becomes impossible to elicit a reflex to stretching in such a piece. Therefore, it is evident that Straub was able to observe the tension reflex because he left the intermyal neurons intact, while Budington obtained a negative result because he took care to remove them.

[5] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915, xxxix, 139.
[6] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915, xxxix, 146.
[7] Knowlton, F. P., and Moore, A. R., *Am. J. Physiol.*, 1917, xliv, 490.
[8] Dawson, A. B., *J. Comp. Neur.*, 1920–21, xxxii, 155.
[9] Straub, W., *Arch. ges. Physiol.*, 1900, lxxix, 379.
[10] Budington, R. A., *Am. J. Physiol.*, 1902, vii, 155.
[11] Bovard, J. F., *Univ. California Pub. Zool.*, 1918, xviii, 135.

It turns out then that the peristaltic waves in Friedländer's experiment are initiated both by sensory stimulation of the integument and by stimulation of the proprioceptors of the muscle layer as the result of passive tension of the myodermal sheath. Each mechanism furnishes a reinforcement of the other and makes the essential peristalsis doubly sure.

II.

Since the musculature of the earthworm contains receptors adequate to initiate reflexes, the possibility arises that such neurons play a part in the coordination of bodily movements. Morgulis[12] has described experiments by means of which he proved that passive deflection of the posterior part of an earthworm results, when locomotion is resumed, in a turning of the anterior segments in a direction parallel to the enforced orientation of the posterior part. The reaction was demonstrated by arranging a worm so that the anterior half rested on a table and the posterior half on a movable piece of slate. Movement of the slate to the right or left caused corresponding alterations in the orientation of the anterior segments of the worm. The result in all cases was a movement of the head segments in such fashion that the line of progress was parallel to the orientation of the posterior segments. Morgulis did not attempt to analyze this phenomenon as to receptors, effectors, and neural paths.

That the effect described is the result of reflexes arising from unequal tension of the musculature rather than from cuticular stimulation is proven by the fact that specimens in which the sensory cells of the epidermis have been anesthetized with $MgCl_2$ give the characteristic response of the head segments when the posterior half of the worm is bent. This is true when the anterior half of the worm is anesthetized, or the posterior half, or the entire worm. In such cases the response is more sluggish than in the normal animal but is none the less definite. The receptors for the reaction therefore lie within the muscular wall. We are thus dealing with proprioceptors similar in function to those discussed in the first part of this paper; it is difficult to escape the conclusion that the receptors in the two cases are the same.

[12] Morgulis, S., *J. Comp. Neur. and Psych.*, 1910, xx, 616.

Furthermore, it can be shown in this experiment that the path of transmission of the impulse forward is through the ventral nerve cord, since section of the cord anterior to the zone of flexion prevents the response from taking place. The reaction can be described by the statement that passive tension of the musculature on one side of the body induces active contraction of the musculature of the same side, forward of the point under tension.[13] Since the result of the reflex is to bring the course of progress into conformity with the new position assumed by the tail, we may refer to it as a *homostrophic* reflex and the innervation concerned as *homostrophic* innervation.[14]

The next point to be determined is the distribution of the receptor system of the homostrophic reflex in the earthworm. To this end the region of passive tension was carried forward as far as practicable; namely, to the 5 to 7 segments, with the result that segments 1 to 3 showed the characteristic response. It is clear, therefore, that this receptor system extends throughout the length of the animal. The same is not, however, true of the corresponding effector mechanism. Removal of the cerebral ganglia or amputation of the anterior segments up to 15 does not interfere with the response, but removal of the anterior 20 segments destroys it for a period of about 2 weeks, after which the reflex returns. The effector system is consequently confined to the anterior 15 to 20 segments—the part of the animal which corresponds in a general way to the head and neck region of the vertebrates. It is not without interest in this connection that a limited number of anterior segments constitute a morphological head in the annelids, since it has been shown that only a certain number of head segments can regenerate. The number is characteristic for each species and varies in the phylum from 4 in *Allolobophora fœtida* to about 15 in *Criodrilus*.[15]

It has doubtless occurred to the reader that the tension reflex of the annelid described above bears a striking resemblance to similar

[13] In reversed locomotion passive unilateral tension of the head segments was without effect upon the direction of progress.

[14] From the Greek noun στροφή meaning a twist or turn as of the body in dancing or wrestling, and the Greek prefix ὁμο- signifying the same; hence *homostrophic* meaning twisting or turning which maintains progress in the same direction.

[15] Hyman, L. H., *J. Exp. Zool.*, 1916, xx, 125.

reflexes exhibited by the neck and body muscles of the vertebrates in their effect on the tension of the eye muscles. The reaction was first observed by Lyon[16] in the dogfish. He showed that if the head of *Mustelus* be held immovable while the tail is bent to the right, the result is a forward rotation of the right and a backward rotation of the left eyeball. In other words, the eyes move as if the animal were to run a course parallel to the position of the tail. This, it will be noted, is exactly analogous to the body positions assumed by the earthworm as the result of unilateral tension. Lyon further found that the reflex passed forward by way of the spinal cord, and that fin movements were not affected by passive tension of the body musculature. De Kleyn[17] has demonstrated the relation in rabbits between passive tension of the neck muscles and compensatory rotation of the eyes. He also has shown that the cervical nerves function as paths for the impulse and that the reflex occurs unvaryingly only in case the semicircular canals are removed. The fact that the eye muscles can be affected simultaneously by the homostrophic reflex resulting from bending the head, and stimulation of the semicircular canals by rotation of the animal on a turntable, has been demonstrated for rabbits by Maxwell, Burke, and Reston.[18] The experiments proved that the effect on eye movements was equal to the algebraic sum of the two forces—a clear case of the interaction of geotropism and the homostrophic reflex.

To summarize, it may be said that the homostrophic reflex plays a subordinate rôle in the orientation of the more highly differentiated forms, the greater part of the work of determining the position in space being taken over by the semicircular canals, statocysts, or light receptors. In earthworms, where geosensitive organs are absent, this form of muscular coordination must be a device of the first importance. As to the question of the relation of the homostrophic reflex to tropisms, it can be said that in general they are the complements of each other. The tropisms constitute a class of responses of the organism to external forces and the homostrophic

[16] Lyon, E. P., *Am. J. Physiol.*, 1900–01, iv, 77.

[17] de Kleyn, A., *Arch. néerl. physiol. homme et animaux*, 1917–18, ii, 644.

[18] Maxwell, S. S., Burke, U. L., and Reston, C., *Am. J. Physiol.*, 1921–22, lviii, 432.

reflex is the reaction of the animal to its own internal tensions. Consequently every turn of the body in response to a momentary external force carries with it its own correction, and the animal continues its course without great deviation. For this reason any permanent alteration of the path by reason of tropistic orientation of the earthworm can occur only when the exciting cause is a force constantly applied.

It also may be not without significance for psychology that in a comparatively simple form, such as an annelid, muscle tension profoundly affects the processes of the central nervous system, and that such tensions exert a determining effect upon a major activity of the animal—locomotion.

## SUMMARY.

1. By the use of preparations of earthworm in which the cutaneous receptors have been anesthetized with a solution of M/8 $MgCl_2$, it is shown that peristalsis can be initiated by tension alone.

2. The receptors of the tension reflex are the intermyal sensory cells of the ventral region of the body wall.

3. It is concluded that Straub obtained the tension reflex because his preparations contained the intermyal receptors; Budington was unable to observe the tension reflex in any preparation from which the intermyal receptors had been removed.

4. Intermyal receptors are the receptors of the following reaction: Passive unilateral tension of the posterior part of an earthworm induces active homolateral tension of the musculature of the anterior segments, and results in the course of progress being brought into line with the enforced orientation of the tail. This reaction is termed the *homostrophic* reflex.

5. The receptors for the reaction are distributed throughout the entire length of the worm, the effectors are limited to the anterior 15 to 20 segments. The impulse is conducted by the ventral nerve cord.

6. The interaction of the homostrophic reflex and tropisms is considered.

# A STUDY OF THE EQUILIBRIUM BETWEEN THE SO CALLED "ANTITRYPSIN" OF THE BLOOD AND TRYPSIN.

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It has been a well known fact for some time that blood serum has the property of retarding the digestive activity of trypsin. We have, however, no satisfactory information regarding either the nature of the substance which effects the retardation or the type of chemical reaction involved in the process. At one time the inhibitory agent was thought to be an immune antibody and in accordance with custom it was called antitrypsin. There is no evidence to support the idea that the active agent is of the nature of an immune body, so the term antitrypsin is incorrect.

Landsteiner (1) found that the retarding effect was associated with the albumin fraction of serum. Hedin (2, 3) then employed preparations of serum albumin as "antitrypsin" in one of the few experimental inquiries that have been made with the view of studying the nature of the trypsin-antitrypsin reaction (4). While these studies were in progress, Hedin[1] (5) discovered that charcoal adsorbed trypsin and that the equilibrium between these two substances satisfied the conditions demanded by the equation representing adsorption phenomena. A comparison of the experimental results obtained when retardation was effected by charcoal with those of the experiments when serum albumin was used presented so many similarities that Hedin was led to the conclusion that the equilibrium between trypsin and "antitrypsin" (serum albumin) was also governed by adsorption phenomena; that is, the trypsin was adsorbed by the serum albumin as it was by the charcoal. It was, however, not possible to obtain values from the experiments to substitute in the adsorption equation. They seemed plausible, though, because

[1] Hedin (3), p. 495.

both reactants were known to be colloids and it was the accepted belief then that the behavior of colloids in solution is governed chiefly by adsorption phenomena. Also, in accordance with this general idea, adsorption was regarded as an essential factor in all enzyme reactions. However, the correctness of Hedin's interpretation concerning the nature of the equilibrium between serum albumin and trypsin, as well as the fundamental assumptions upon which they are based, are questioned by experimental results recently obtained in a study of the mechanism of the inhibiting action of other substances on trypsin, pepsin, and invertase.

In the experiments referred to, the equilibrium between trypsin and the inhibitory substance formed by the action of the enzyme on protein was studied. Gelatin was hydrolyzed by trypsin until no further increase in formol titration was noted and a solution was made from the products formed. A method was developed by means of which the amount of retardation caused by the inhibiting agent in the solution could be measured quantitatively (6). The retardation effected by the inhibiting solution was accounted for by the conception that the trypsin and the active inhibiting agent combine to form a compound that is inactive. The compound thus formed dissociated so that part of the original trypsin added was combined and inactive and part free or active. In order to subject the reaction between trypsin and the inhibiting substance to analysis it was assumed that the equilibrium for the compound formed by the combination of these substances is governed by the law of mass action and that the rate of hydrolysis is proportional to the concentration of free enzyme. An equation derived from the law of mass action was formulated to express the experimental results to be demanded if the assumptions made were correct. Experiments were made (7) in which each component entering the reaction was varied independently, and the results observed were in such close agreement with those calculated from the equation developed that the assumptions were satisfactorily supported. These results are taken as evidence which supports the view that an enzyme reaction may be explained by conceptions other than those embracing adsorption theories. To assume the equilibrium studied to be governed by surface phenomena, it would be necessary to suppose that the tryp-

sin adsorbed the inhibiting agent, since there is no evidence that this is present in other than true solution. Moreover, the results would not lend themselves to mathematical analysis on such an assumption because the adsorption equation contains no term to represent the amount of adsorbent combined. There would then be no way to determine a value for the trypsin combined and for the free trypsin.

The same mechanism described for trypsin was found in the case of pepsin (8). In this case, the combination was between pepsin and the digestion products formed during the time pepsin was acting on the protein. Further evidence that enzyme reactions may conform to the same principles is found in the work of von Euler and Svanberg (4). These investigators have shown this to be so for the equilibrium between invertase and various crystalloidal substances which retard its activity. Inasmuch as it is possible to account for enzyme reactions on the basis of the laws of general chemistry, there seems to be no theoretical reason to disregard the fact and seek explanations in the adsorption theories. Further, as to Hedin's observation regarding the adsorption of trypsin by charcoal, it is perfectly possible that trypsin may be adsorbed by charcoal and yet react with other substances according to the principles stated in the mass action law. Such instances are known in general chemistry.

With the information supplied by these facts, together with theoretical considerations based on them, it has seemed important to make further inquiries about the equilibrium concerned in the retardation of tryptic activity by constituents in the blood. In addition to these considerations, a better understanding of the nature of this reaction is of importance in pathology since the inactivating property of blood serum for tryptic digestion has engaged the interest of several investigators in this field for a number of years. It has been claimed that this property of serum varies with respect to certain diseases and also that it bears an important relation to immune states of the human body. In this communication we will report the results of an investigation planned to determine whether or not this inhibiting effect can be accounted for by the same mechanism as has been found to hold in the case of the other inactivating substances already mentioned. Such an investigation is made possible by having at our disposal a method which permits a quantitative determination

of the amount of trypsin present in a digestive mixture during the course of hydrolysis. This method has been described in a previous paper (9).

In the paper referred to, in which we described the method we employed in the present investigation, it was pointed out that the time required to cause a given change in the viscosity of a gelatin-trypsin solution was inversely proportional to the amount of trypsin present. This fact then permits the use of that value $\frac{1}{(\text{T hours})}$ as a measure for the enzyme. For the observations to be reported we chose 20 per cent change in viscosity as the experimental end-point. As an arbitrary standard for a unit of trypsin we adopted that amount required to change the viscosity of the gelatin solution 20 per cent in 1 hour. The gelatin solutions used in all the experiments contained 3 per cent dry weight of gelatin and were adjusted to pH 7.4. These solutions were heated to 50°C., then placed in a water bath, and kept at 34°C., where they remained until used for an experiment. The interval was about 14 to 16 hours. The trypsin solutions were prepared as previously described (7). We have employed plasma instead of serum as the inhibiting substance. This was done at first because we were using plasma for other experiments and later we found that the inhibiting substance was much more stable in plasma than in serum. This fact will be investigated at another time. The plasma was obtained from rabbits as previously described (10). A 1:10 or a 1:20 dilution of plasma was found to give satisfactory inhibition.

The first experiment was made to determine whether we could measure the amount of retardation quantitatively. In the same experiment we were also able to obtain information regarding the uniformity of the retarding effect of plasma obtained from different animals. 1:20 dilutions of four different samples of plasma were made and equal volumes of these were mixed with equal volumes of a 2:25 dilution of trypsin solution. 0.5 cc. of this mixture was added to 25 cc. of gelatin of concentration and reaction as previously stated, then after thorough mixing, 10 cc. of the mixture were pipetted into separate viscosimeters and observations on the time of outflow were made at frequent intervals. In another viscosimeter there

were 10 cc. of gelatin which contained the same amount of trypsin but with no inhibitor. If the amount of retardation can be measured quantitatively we should find that the inhibition is practically independent of the progress of the change in viscosity over the range selected for our experiments. The results tabulated in Table I show that this is the case within the limits of precision the method

TABLE I.

*Effect of Plasma on Retarding the Activity of Trypsin.*

| Change in viscosity. | $T$ hrs. for 20 per cent change in viscosity. | | $\frac{T \text{ inhibitor}}{T \text{ control}}$ |
|---|---|---|---|
| | Control. | Plasma. | |
| *per cent* | | | |
| 5 | 0.08 | 19.5 | 2.44 |
| 10 | 0.18 | 44.0 | 2.44 |
| 15 | 0.31 | 75.0 | 2.41 |
| 20 | 0.52 | 115.0 | 2.21 |

TABLE II.

*Effect of Retardation by Plasma from Different Individuals.*

25 cc. of 3 per cent gelatin of pH 7.4 + 0.5 cc. of trypsin-inhibitor mixture or trypsin-water mixture for control. 10 cc. pipetted into a viscosimeter.

| | $T$ hrs. for 20 per cent change in viscosity. |
|---|---|
| 1. Control: Gelatin { trypsin 2:25 dilution, 0.25 cc. / water, 0.25 cc. } | 0.52 |
| 2. Plasma 1: Gelatin { trypsin dilution, 0.25 cc. / plasma 1:10 dilution } | 1.20 |
| 3. " 2: " " " | 1.15 |
| 4. " 3: " " " | 1.19 |
| 5. " 4: " " " | 1.15 |

allows. In Table II the effect of retardation caused by plasma from different individuals is shown. It will be noted that there is apparently good uniformity.

In the studies concerning the equilibria between the enzymes and inhibitory agents already referred to, it was found in each case that the equilibrium was reached practically instantaneously. Because

of this fact the experimental result was not influenced by the order of mixing the reactants or by the length of time they were allowed to stand. It is important then that we obtain data in this connection for the equilibrium with which our study is concerned; that is, whether there is a difference in the experimental result when we first mix the dilutions of trypsin solution and plasma, then add the mixture to gelatin, or when the diluted plasma is first added to the gelatin, followed by the trypsin solution. If the equilibrium under consideration is similar to the charcoal-trypsin equilibrium, we would expect to find different results since in this case the equilibrium is not reached until some time after the reactants are brought together. It is also

TABLE III.

*Influence of Order of Mixing.*

| | $T$ hrs. for 20 per cent change in viscosity. | $Q = \frac{1}{T}$ hrs. |
|---|---|---|
| 50 cc. gelatin + { trypsin 2:25 dilution, 0.5 cc. / water, 0.5 cc. } (control.) | 0.87 | 1.15 |
| 50 " " + { trypsin 2:25 dilution, 0.5 cc. / plasma 1:20 " 0.5 cc. } | 1.30 | 0.77 |
| (50 cc. gelatin—plasma 1:20 dilution, 0.5 cc.) + trypsin dilution 2:25, 0.5 cc. | 1.36 | 0.75 |

not easily reversible. Hedin, as previously stated, found that the equilibrium between trypsin and "antitrypsin" was influenced by the time of standing, and this result was accepted as evidence to indicate an analogy between the behavior of charcoal and "antitrypsin."

The results of the first experiment made showed a difference when the order of mixing was different. The difference was a little greater than our allowable experimental error. This was true in the second experiment but the difference was reversed. Because of the importance of the information sought, we repeated the experiment several times and while differences were observed, there was no regularity with respect to the order of mixing. Ten such experiments were made and the data obtained from the tenth experiment are given in Table III. It will be noted that within the precision of our method

the order of mixing does not influence the experimental result. However, it is much more difficult to add the plasma and trypsin solutions separately, and for this reason further experiments to be reported were made by first mixing the plasma and trypsin. Table IV shows the results of an experiment made to determine the influence of time of standing on the inhibiting effect. This factor too is shown to be of no importance, since the ratio T inhibitor : T control remains practically constant. Our conclusion then is that the equilibrium under consideration is one that is reached practically instantaneously and is rapidly and completely reversible, as was found to be the case with the equilibria already mentioned.

TABLE IV.

*Influence of Time of Standing.*

Equal volumes of trypsin, dilution 2:25, and plasma, dilution 1:20, mixed, then 0.5 cc. of this mixture added to 25 cc. of gelatin at intervals noted.

| | Time to change 15 per cent viscosity. | | $\frac{T \text{ plasma}}{T \text{ control}}$ |
|---|---|---|---|
| | After | | |
| | 0 hrs. | 2.5 hrs. | |
| Control. | 0.48 | 0.66 | 1.37 |
| Test. | 0.78 | 1.03 | 1.32 |

This experiment is a critical one for identifying the nature of the reaction since adsorption or other reactions in a two-phase system are as a rule only partially or slowly reversible. On the other hand, reactions in homogeneous solutions are usually easily and rapidly reversible, in accordance with the law of mass action. We shall not attempt to develop a further discussion of the difference between our results and those obtained by Hedin. The experimental conditions in each case are entirely different. For example, Hedin's observations have to do with changes found in equal times, the digestion was allowed to proceed for 24 hours at 37°C., and the enzyme preparations were not the same. This method of procedure leads to confusion in the results with respect to the influence of spontaneous inactivation of the enzyme and of the inhibitive agent (11).

The observations so far stated make it appear that there is striking similarity between the behavior of the inhibiting agent contained in plasma and that contained in the other substances referred to. It now remains for experiments to be made which will further analyze the equilibrium under consideration. Three experiments were

TABLE V.

*Experiments in Which the Plasma is Varied and the Trypsin Kept Constant.*

25 cc. gelatin 3 per cent, pH 7.4.

0.25 cc. trypsin, 2:25 dilution; 0.25 cc. plasma, 1:10 dilution.

$K = 0.17$ $I = 485$ $E = 2.2$

| Plasma $\times 10^{-3}$ $P$. | $T$ hrs. for 20 per cent change in viscosity. | Trypsin. | | | |
|---|---|---|---|---|---|
| | | Observed free $Q=\frac{1}{T}$ hrs. | $Q$ calculated. | Combined $E-Q$. | Inactivated. |
| *cc.* | | | | | *per cent* |
| 0.0 | 0.45 | 2.2 | (2.2) | — | — |
| 0.63 | 0.69 | 1.45 | 1.40 | 0.75 | 34.0 |
| 1.25 | 1.05 | 0.95 | 1.01 | 1.25 | 37.0 |
| 2.5 | 1.55 | 0.65 | 0.64 | 1.55 | 71.0 |
| 5.0 | 2.10 | 0.47 | 0.45 | 1.72 | 79.0 |

TABLE VI.

*Experiments in Which the Trypsin is Varied While the Plasma is Kept Constant.*

$K = 0.17$ $I = 275$ $P = 0.025$

| Trypsin $\times 10^{-2}$ | $E$ | $T$ hrs. for 20 per cent change in viscosity. | Trypsin. | | | |
|---|---|---|---|---|---|---|
| | | | Observed free $Q=\frac{1}{T}$ hrs. | $Q$ calculated. | Combined $E-Q$. | Inactivated. |
| *cc.* | | | | | | *per cent* |
| 6.25 | 5.84 | 0.31 | 3.23 | 3.05 | 2.61 | 44.8 |
| 3.15 | 2.92 | 0.74 | 1.35 | 1.31 | 1.57 | 53.8 |
| 1.56 | 1.46 | 1.60 | 0.62 | 0.60 | 0.94 | 60.6 |
| 0.78 | 0.73 | 3.64 | 0.275 | 0.29 | 0.46 | 62.4 |

planned for this end: namely, the effect of adding increasing amounts of plasma to a constant amount of trypsin; the effect of varying the trypsin while the plasma was kept constant; the effect of varying the volume, *i.e.* the effect of dilution on the trypsin-plasma mixture.

The results of these experiments are given in Tables V, VI, and VII, and their graphic representation is shown in Figs. 1, 2, 3, and 4.

TABLE VII.

*Effect of Diluting a Mixture of Trypsin and Inhibiting Substance.*

$K = 0.17$ $I = 70$ $E = 20$

| Volume. | Time in hrs. for 20 per cent change in viscosity. | Trypsin. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Observed active $\frac{1}{T}$ hrs. | | Calculated. | | Combined. | Inactivated. | |
| | | Total. | Per 25.5 cc. | Total. | Per 25.5 cc. | Observed. | Observed. | Calculated. |
| *cc.* | *hrs.* | *units* | *units* | *units* | *units* | *units* | *per cent* | *per cent* |
| 25.5 | 0.065 | 15.40 | 15.4 | 16.4 | 16.4 | 4.60 | 23.0 | 18.0 |
| 51.0 | 0.118 | 16.94 | 8.47 | 17.1 | 8.55 | 3.10 | 15.3 | 14.5 |
| 102.0 | 0.220 | 18.20 | 4.55 | 17.8 | 4.50 | 1.80 | 9.0 | 11.0 |
| 204.0 | 0.415 | 19.3 | 2.41 | 19.1 | 2.50 | 0.70 | 3.5 | 4.5 |

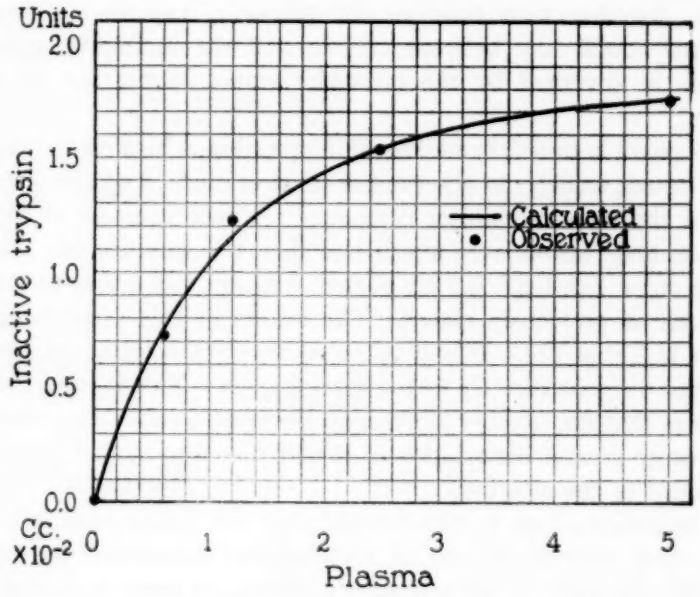


FIG. 1. Inactivation of trypsin caused by increasing quantities of plasma.

The curve in Fig. 1 represents the effect of adding increasing amounts of plasma to a constant quantity of trypsin. In this graph the units of combined trypsin are plotted as ordinates against the cubic centimeters of plasma added as abscissæ. The slope of this curve increases very rapidly at first, then approaches an asymptote. This demonstrates the fact that the first unit amount of plasma added has a much greater inhibitive effect than subsequent unit amounts added. These results, though suggestive of reactions in qualitative agreement with the principles of the mass action law, could be explained equally well by other considerations.

In the second experiment where varying amounts of trypsin are added to a constant amount of plasma, we would expect to find either the percentage inactivation equal for each amount of trypsin or increasing with each decrease in the amount of trypsin. The data for this experiment are given in Table VII. The percentage inactivation is observed to increase with smaller amounts of trypsin. These results, together with those of the preceding experiment, suggest that the equilibrium between trypsin and the inhibiting agent in plasma is governed by the principles stated in the law of mass action.

It is possible to obtain additional information to bear on the suggestions stated; namely, to observe the effect of dilution on the dissociation of the trypsin-inhibitor compound. Equal volumes of trypsin solution and plasma were mixed and then this mixture was diluted in the order 1–2–4–8. 0.5 cc. of each dilution was added to gelatin solutions as previously stated. The results of this experiment are shown in Table VII. It will be noted that the total active trypsin does not decrease directly as the total trypsin. This is experimental evidence that the effect of dilution on the trypsin-inhibitor compound is to cause greater dissociation of it and therefore more free trypsin is present in the solution.

In summary, then, it may be said that the results obtained from these three experiments furnish evidence that strengthens the suggestion that the equilibrium between the inhibitory agent in plasma and trypsin is similar in behavior to the equilibria between the enzymes and inhibitory agents mentioned earlier in this paper. It now remains for us to determine how the experimental results can be calculated.

*Formulation of Results.*

In attempting to calculate the experimental results only one equation was found which would fit the curves for all three experiments. This equation was formulated from the assumption that the results obtained from our experiments indicate that one molecule of trypsin combines with one molecule of inhibiting agent to form one molecule of trypsin-inhibitor compound; that is, the reaction in equilibrium may be represented by the expression

$$\text{Trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin-inhibitor}$$

In order to determine the quantitative relation between these reactants in equilibrium the chemical expression can be put into a form for mathematical treatment. This is accomplished in the following equation where the reactants are expressed in terms of their concentrations.

$$\frac{\text{Concentration of free trypsin} \times \text{concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

If now we let

$E$ = the total trypsin
$Q$ = the free trypsin
$I$ = the units of inhibitor in $P$ cc. of plasma

we may substitute these symbols and write the equation

$$\frac{\left[\frac{Q}{V}\right]\left[\frac{PI-(E-Q)}{V}\right]}{\left[\frac{E-Q}{V}\right]} = K$$

This simplifies to

$$\frac{[Q]\,[PI-(E-Q)]}{V\,[E-Q]} = K \tag{1}$$

Solving this equation for $Q$ we obtain

$$Q = \sqrt{\left(\frac{PI+KV-E}{2}\right)^2 + KVE} - \frac{PI+KV-E}{2} \tag{2}$$

We can obtain values from our experimental results for all the terms in equation (1) except $K$ and $I$. It is possible, however, to get a solution of approximation by assuming values for $I$, then solving

for $K$. When this is done it is found that over a certain range of values $K$ is negative, another range of values gives $K$ positive values, but when these values are substituted in equation (2) the calculated results differ widely from the observed results. There is still another range of values for $I$ that give positive values for $K$ which yield a satisfactory agreement with the observed results. If the same value of $I$ is used for all three experiments one obtains a different $K$ in each instance. These values increase in the order of the experiments. We know that the inhibitive action of plasma decreases with time and this necessarily affects the value of $I$. It follows then that the value of $I$ must be different in each experiment; that is, the change in the inhibitive agent is quantitative rather than qualitative. It would be unreasonable to assume that the value of $K$ would actually vary, and the order of the variation found for the same value of $I$ is what might be expected; namely, that $I$ has the highest value in the first experiment and the lowest value in the third or last experiment. It is necessary, therefore, to take a fixed value for $K$, then substitute in equation (1) and solve for the value of $I$ in the different experiments. We accordingly took one of the values of $K$ determined for the second experiment. The $K$ chosen was that one calculated with an assigned value of $I$ which gave the most constant values of $K$ in the four observations of the experiment. The reason for selecting a $K$ from the second experiment will appear later. The values found for $I$ by this procedure decrease in the order of the experiments as follows: For the first experiment $I = 485$, for the second experiment $I = 275$, and for the third experiment $I = 70$.

At this time we are not prepared to discuss the conditions governing the spontaneous destruction of the inhibitive agent so we are not in a position to comment on the significance of the relative differences stated for $I$. Some indication of these relations can be formed, however, for the first and second experiments. From the curves in Figs. 2 and 3 it will be observed that for two units of trypsin, double the amount of plasma is required in the second experiment to combine the same amount of trypsin. This agrees almost exactly with the relative values of $I$ found by calculation and is strong evidence that the values of $I$ are not mere calculated figures.

The value of $K$ was taken from the second experiment because equation (1) was found to be the only expression that satisfied the curve (Fig. 3) for this experiment. The curves representing Experiments 1 and 3 (Figs. 2 and 4), however, may be satisfied by several equations. This is due to the fact that in these experiments the amount of inhibitor combined is small as compared with the amount that is free. As a consequence the term $(E-Q)$ is negligible with respect to the term $(PI)$. This fact allows the term $(E-Q)$ to be neglected in the

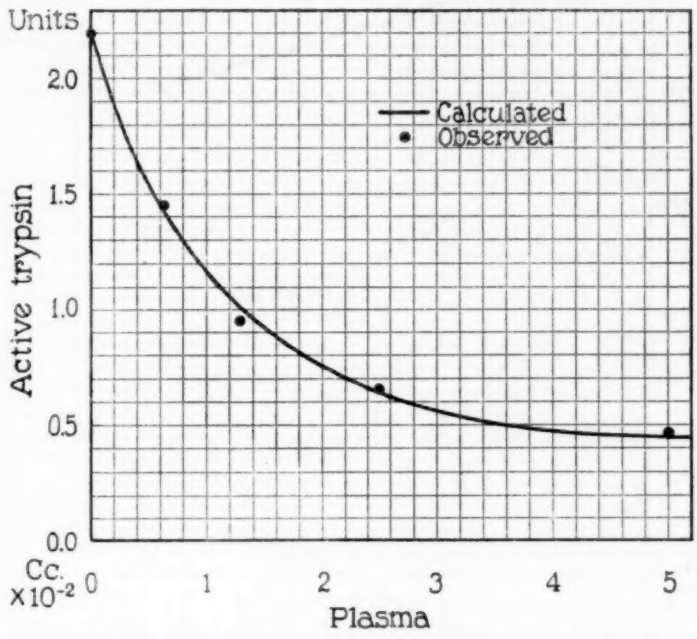


FIG. 2. Inactivation of trypsin caused by increasing quantities of plasma.

numerator of equation (1), and this in turn permits one to obtain reduced forms of the equation which, when solved for $Q$, satisfy the curves for Experiments 1 and 3. The same conditions do not hold in Experiment 2; therefore, this simplification cannot be made.

In presenting the mathematical analysis of the experiments described, we realize that we have employed an equation containing two arbitrary constants. We are cognizant of the bearing this fact might have on the significance of our calculated results.

We feel justified, however, in accepting the agreement between the calculated and observed results as significant, since only one equation was found that fits the curves for all three experiments. Also in

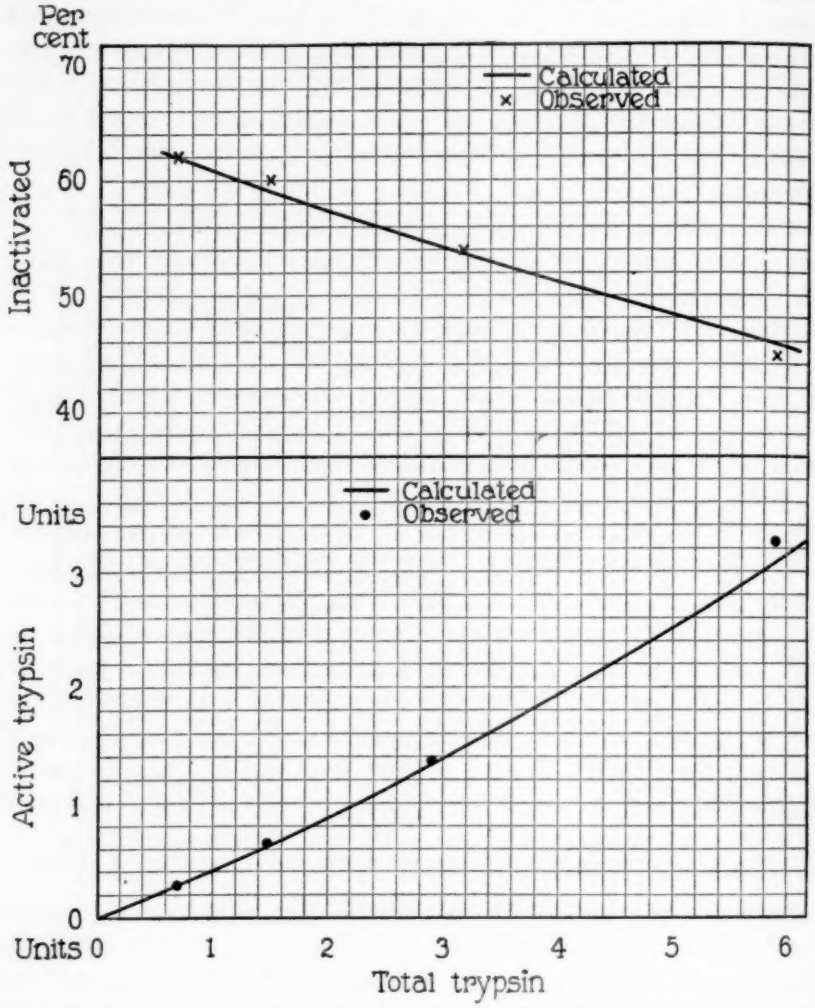


FIG. 3. Effect of a constant quantity of plasma on varying quantities of trypsin.

obtaining the calculations it was necessary to use different values of $I$ which decrease in order from one experiment to the other, as would be expected from the known fact that the inhibitive action of

plasma decreases with time. Since the equation used is derived from the mathematical expression of the law of mass action, these considerations lead us to conclude that we have presented experimental evidence which, though not final, is more than just suggestive

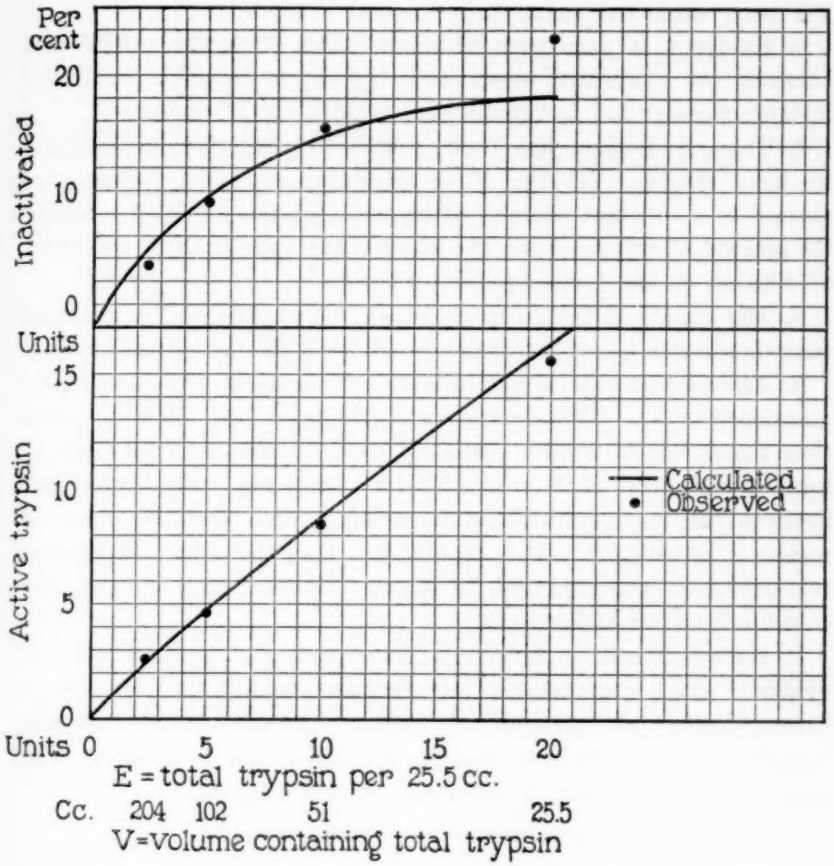


FIG. 4. Effect of dilution on a mixture of trypsin and plasma.

that the following statements are true: (1) the inhibition of tryptic activity by plasma is effected by an agent which combines with trypsin to form an inactive compound; (2) this compound is dissociated into free trypsin and free inhibitor; (3) the conditions of equilibrium for the reaction are governed by the law of mass action.

## CONCLUSIONS.

1. The retarding effect of plasma on the action of trypsin can be measured quantitatively.

2. The nature of the reaction involved in effecting the retardation has been subjected to an experimental study.

3. Evidence is presented which indicates that the equilibrium between the inhibitive agent and trypsin is reached practically instantaneously and is rapidly and completely reversible.

4. This equilibrium has been studied by experiments in which we have observed (1) the effect of adding increasing amounts of plasma to a constant amount of trypsin, (2) the effect of varying the amount of trypsin while the plasma was constant, (3) the effect of dilution on the trypsin-plasma mixture.

5. The results of these experiments are discussed and it is stated that they are in quantitative agreement with the law of mass action.

6. An equation was found which fits the curves for the experiments mentioned in (4). This equation was developed from the assumption that 1 molecule of trypsin combined with 1 molecule of inhibitor to form 1 molecule of trypsin-inhibitor compound. The agreement between the results calculated by this equation and the observed results is satisfactory. It is pointed out that the equation contains two arbitrary constants and the bearing this fact may have on the calculated results is discussed.

7. We conclude from the results of our study that we have adduced evidence which suggests the following statement regarding the so called "antitryptic" property of blood. The inhibitive agent and trypsin combine to form an inactive but dissociable compound. The reaction in equilibrium is expressed by the equation

$$\text{Trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin-inhibitor}$$

The conditions of equilibrium are apparently governed by the law of mass action. The behavior of the equilibrium is therefore similar to the behavior of other equilibria between different inhibitive agents and enzymes discussed in the paper.

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# A METHOD FOR THE QUANTITATIVE DETERMINATION OF TRYPSIN AND PEPSIN.

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In the study of proteolytic enzyme reactions an experimental difficulty has always presented itself in the lack of a method permitting the determination of the amount of enzyme present in a given digestion mixture during the course of the reaction. This difficulty has been overcome by a method already described which depends on the change in electrical conductivity during the digestion of egg albumen by pepsin (1), and during the digestion of gelatin by trypsin (2). This method has been employed successfully in a series of experimental studies (2, 3).

While this procedure has been found useful, there are certain studies concerning enzyme reactions in which it cannot be employed. This is particularly true where it is desired to study these reactions when they take place in the presence of "buffer" salts. It occurred to one of the writers that the rate of change in viscosity of gelatin during digestion might be directly related to the amount of enzyme present in the digestion mixture. If this idea is correct, one would expect to find that the time required to cause an equal percentage change in the viscosity of a series of gelatin solutions would be directly proportional to the amount of enzyme added, provided the gelatin solutions are the same but the amount of enzyme in each is varied. That this actually is found to be the case will be shown in this communication.

In order to test the idea stated, it is necessary to consider certain factors which *per se* bring about changes in the viscosity of gelatin solutions; namely, the temperature at which the observations are made, the reaction, and the salt concentration of the solutions. The observations of Loeb (4) regarding the effect of pH and the concen-

tration of salt, and the observation of Loeb and others concerning the effect of temperature, demonstrate the influence of these factors on this property of gelatin. Obviously, then, it is necessary to control these conditions during observations where changes in viscosity are assumed to depend directly upon enzyme action. Further, in the case of experiments on trypsin digestion, the influence of temperature on the stability of the enzyme must be considered.

Fortunately, as far as temperature effects are concerned, we know that a temperature of 34°C. can be used without affecting the stability of trypsin to an extent prohibitive for the conditions of our experiment (2). It so happens that this same temperature is the one at which the minimum thermal effects on viscosity are observed. Regarding the influence of pH, we know, as a consequence of Loeb's observations, that the slope of the curve representing viscosity changes in gelatin solutions as a function of the pH approaches zero and is sensibly constant between pH 7.3 and 7.5. The addition of a "buffer" salt on the alkaline side of the isoelectric point of gelatin easily corrects for any pH effect, but the concentration of the salt used must be one that will not develop a degree of viscosity which will make the time of outflow for the solution too long. On the acid side of the isoelectric point the reaction can be controlled by adding a weak acid, such as $H_3PO_4$. We have found it possible by these means to prepare gelatin solutions which meet all the requirements demanded for observations to be made on either side of the isoelectric point.

*Preparations for Experimental Procedure.*

The gelatin employed was Cooper's powdered preparation which was brought to the isoelectric point and washed as described by Loeb (4), except that larger amounts were made. The isoelectric gelatin was melted and the concentration of gelatin determined by dry weight. A given amount of gelatin was kept at a temperature between 100°C. and 102°C. for 24 hours for this determination. For trypsin digestion the concentrated gelatin preparation was diluted with 0.1 M $Na_2HPO_4$ so as to contain 3 gm. of gelatin by dry weight per 100 cc., then it was adjusted to pH 7.4 with 0.1 M $H_3PO_4$. For pepsin digestion the concentrated gelatin was adjusted to pH 3 with dilute phosphoric acid and diluted to contain 3 gm. of gelatin per 100 cc. The diluted gelatin was in each case distributed in 250 cc. amounts in Erlenmyer flasks and stored in a refrigerator kept at 3°C. A small amount of thymol was added to each flask as a preservative.

The enzyme solutions were prepared as previously described by Northrop (2, 3).

The viscosimeters were made after the pattern of the Ostwald type and were calibrated by regulating the time of outflow of 5 cc. of water at 34°C. to be 60 seconds between two marks. The gelatin-water time ratio was approximately 3. The time of outflow was measured by means of stop-watches. The viscosimeters were held in a stand which was kept constantly in a deep water bath. The viscosity obtained in this way is the mean viscosity over the period of time elapsed during the measurement. For convenience in plotting, however, the value is assumed to represent the viscosity at the center of this time interval. The error introduced by this method is negligible.

## EXPERIMENTAL RESULTS.

For trypsin digestion 50 cc. of 3 per cent gelatin, pH 7.4, were pipetted from a flask that had been in the water bath at 34°C. over night and put into an empty flask also immersed in the water bath. 0.5 cc. of trypsin dilution was then added. After thorough mixing, 10 cc. of this gelatin-trypsin mixture were pipetted into each of 5 viscosimeters and the time of outflow was observed at frequent intervals. The time intervals of observations were based on hundredths of the hour for convenience and were measured from the time of addition of the trypsin. This procedure was carried out with four different dilutions of trypsin, the relative dilutions were of the order 1–2–4–8. The actual times in seconds required for the digestion mixture to pass between the two chosen points on the viscosimeter were plotted as ordinates against the time intervals of observation as abscissæ; then smooth curves were drawn through these points. The time required to cause the same percentage change in the viscosity of the different solutions in each viscosimeter was then interpolated from these curves. Some difficulty was experienced in determining the zero point for drawing these curves. At first we attempted to use for the zero the number of seconds required for the outflow of gelatin when diluted with a volume of water equal to that of the trypsin dilution used. We found, however, that this point frequently did not fall on the same curve as subsequent readings. In such cases the zero was determined by extrapolating back. Later we found that it was much better to follow this procedure entirely.

The time values obtained by interpolation for each group of dilutions were averaged and the mean of this figure was taken as the time

required to cause the change. A group of these curves for different dilutions in the same viscosimeter is shown in Fig. 1. It was found that the values so obtained varied nearly directly as the reciprocal of the relative amount of trypsin taken; that is, double the amount of enzyme requires half the time to cause the same change (Arrhenius' "QT" rule). In Table I the data obtained from such an experiment are shown. The figures are the average of four determinations. Several similar experiments were made. It will be noted that the method permits the calculation of the quantity of enzyme present

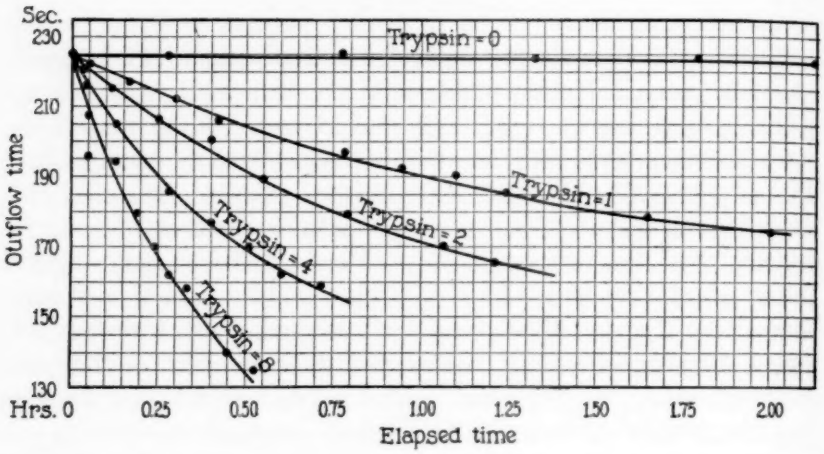


FIG. 1. Effect of increasing quantities of trypsin on the changes in viscosity of gelatin solutions.

with approximately 5 per cent error which we believe to be a satisfactory degree of precision.

One experiment was made to observe whether the method would serve with an equal degree of precision in the case of gelatin-pepsin digestion. Different dilutions of pepsin were mixed with properly adjusted gelatin solutions and these mixtures were run in duplicate. The results obtained, as in the previous experiments, show that the method can be applied satisfactorily in this case.

Another experiment was made to determine whether there was any relation between conductivity changes and viscosity changes during digestion with trypsin. No parallelism was observed between

TABLE I.

*Change Caused in Viscosity of 3 Per Cent Gelatin Solution pH 7.4 by Various Amounts of Trypsin Q. Temperature 34°C. ± 0.02°C.*

| Change in viscosity. | Mean time to cause per cent change noted. (Time interpolated from curve.) When Q = | | | | Value of K. When Q = | | | | Mean for all values of QT. | Q calculated. Mean of all values of QT / Mean time | Q taken. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 1 | 2 | 4 | 8 | | | |
| *per cent* | *hrs.* × $10^2$ | *hrs.* × $10^2$ | *hrs.* × $10^2$ | *hrs.* × $10^2$ | | | | | | | |
| | | | | | | | | | | 0.97±0.05 | |
| 5 | 26.8±0.3 | 12.3±0.4 | 6.5±0.1 | 3.3±0.1 | 26.8±0.3 | 24.6±0.8 | 26.0±0.4 | 26.4±0.8 | 25.9±0.6 | 0.98±0.05<br>0.99±0.06<br>0.94±0.04 | 1 |
| | | | | | | | | | | 2.10±0.12 | |
| 10 | 57.8±0.5 | 27.0±0.9 | 14.3±0.3 | 7.1±0.2 | 57.8±0.5 | 54.0±1.8 | 57.2±1.2 | 56.8±1.6 | 56.5±2.7 | 2.00±0.12<br>2.10±0.13<br>2.05±0.11 | 2 |
| | | | | | | | | | | 4.00±0.10 | |
| 15 | 97.0±0.9 | 45.6±1.5 | 24.2±0.4 | 12.2±0.4 | 97.0±0.9 | 91.2±3.0 | 96.8±1.6 | 97.6±3.2 | 95.7±4.6 | 3.95±0.24<br>3.95±0.21<br>4.01±0.18 | 4 |
| | | | | | | | | | | 7.89±0.38 | |
| 20 | 154.0±2.1 | 74.8±1.9 | 35.5±0.2 | 17.0±0.4 | 154.0±2.1 | 149.6±3.8 | 142.0±0.8 | 136.0±3.2 | 145.5±5.3 | 7.35±0.45<br>7.82±0.35<br>8.55±0.42 | 8 |

these. Since it is probable that the change in viscosity represents the hydrolysis of the gelatin molecule itself while we know the conductivity change to be a function, under the proper conditions of experiment, of the complete hydrolysis, no direct connection between viscosity and conductivity changes would be expected.

The course of the reaction as followed by the viscosity curve is not monomolecular, so it differs from the changes in viscosity taking place during spontaneous hydrolysis as studied by von Schroeder (5).

## SUMMARY.

A quantitative method is described which permits a determination of the relative amount of trypsin or pepsin present in a gelatin-enzyme digestion mixture, provided the gelatin and trypsin solutions are purified.

This method is dependent upon the change in viscosity of such solutions. It is found that the time required to cause a given percentage change in the viscosity is nearly inversely proportional to the amount of enzyme present.

It is pointed out that the particular value of the method lies in the fact that enzyme reactions which take place in the presence of "buffer" salts may be studied.

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4. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.
5. von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 75.

# FURTHER OBSERVATIONS ON THE INFLUENCE OF SALTS WHEN INJECTED INTO THE ANIMAL BODY.

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(Received for publication, November 22, 1922.)

In a previous communication (1) we reported the observation that following the exposure of rabbits to x-rays of relatively long wavelength their plasma bicarbonate is significantly increased. There was apparently some relation between the establishment of this state and the decrease in the circulating mononuclear cells known to follow characteristically after x-ray exposures. This observation led us to inject $NaHCO_3$, in solution, into rabbits and study what influence might be exerted on these same properties of the blood. The results were analogous to those following exposure to x-rays; namely, the pH was increased and there was a marked decrease in the circulating mononuclear cells. The maximum pH increase was reached in about 1 hour and the maximum decrease in the cells was reached in about 3 hours. This experience led us to investigate the effect of other salts when injected into the body. At the time the observations referred to were reported we did not have sufficient data on the latter experiments to warrant their publication. Since then we have carried our observations further.

We continued to use rabbits as experimental animals, and individuals of approximately the same weight (about 2 kilos) were selected. The conditions of feeding and the general care of the animals previously reported (1) were adhered to. Absolute and differential white blood cell counts were made in the usual way. Two or three counts were made before an experiment was begun and any animal whose count showed a significant fluctuation was not used. We believe that we have a sufficient number of standard counts to assure a satisfactory degree of reliability in the counts made following the injections of salt solution. In presenting the changes noted in the mononuclear

cells we have taken the number per cubic millimeter, observed before starting the experiment as 100 per cent, and subsequent counts are expressed in terms of percentage of the original number. Observations made following the injection of 5 cc. of 0.15 M NaCl revealed no significant change in the count. The same observation was made following the injection of 5 cc. of NaCl of the same concentration in 10 cc. volume.

We chose NaCl as the first salt to be injected. Since this is a neutral salt any result observed may be considered to be independent of pH change. The injection of 5 cc. of 2.4 M NaCl solution was followed by a marked drop in the number of circulating mononuclear cells per cubic millimeter of blood. 3 hours after the injection, the number of these cells was 70 per cent below the number present before the experiment was begun. After this time there was a gradual return to approximately the original count. At the end of 24 hours the blood picture was practically that observed before beginning the experiment. This procedure was repeated several times with similar results. This was true whether the injections were made intravenously or intraperitoneally. The same statement applies to all other results to be reported in this paper.

In order to investigate further the relation of pH to the changes observed, we injected solutions of $Na_2HPO_4$ and $KH_2PO_4$. Sörensen salts were employed and solutions of these were made equivalent with respect to the Na ion in the NaCl solutions; *i.e.*, 1.2 M $Na_2HPO_4$ and 2.4 M $KH_2PO$. It would be expected that some change in the pH of the blood would result but in opposite directions. The results observed were identical with those in the experiment where NaCl was injected, and seemed to rule out any influence of pH on the changes we had observed. These observations led us to believe that perhaps we were dealing with ion effects. This was suggested by the fact that the concentration of cations in all the solutions was the same. It was possible to test this idea further by injecting $Na_2SO_4$. We would expect that a 1.2 M solution of this salt would have the same effect as a 2.4 M solution of NaCl. This was found to be the case. It seems unlikely that altered osmotic conditions would play a rôle in the results observed, but in order to have direct information regarding any possible influence of this factor we injected solutions

of cane-sugar and solutions of glycerol. In each case solutions equiosmotic with 2.4 M NaCl were employed; *i.e.*, 4.8 M. We observed no significant change in the number of circulating mononuclear cells. The point of view stated, that we were dealing with ion effects, was encouraged by this observation which we accepted as evidence eliminating osmotic influences. The salts employed so far all furnish ions in common with those that are present in greatest abundance in the blood. It seemed desirable now to determine what effect would follow the injection of a salt with ions not found in significant amounts in the blood. A 2.4 M solution of $LiNO_3$ was injected, and results practically identical with those found in the cases of other salts were observed.

We now made injections of NaCl, $NaHCO_3$, and $LiNO_3$ in 1.2 M and 0.6 M solutions, and $Na_2SO_4$ in 0.6 M and 0.3 M solutions. All of these solutions influenced the blood picture in a similar manner; namely, the mononuclear elements showed a decrease of about 30 per cent instead of about 65 per cent as noted with the higher concentrations of the same salts. No significant difference was noted between the effect of either of these lower dilutions, and this suggests that there is a certain level in the concentration of ions which may be regarded as a threshold point.

Briefly stated, our observations are as follows: When NaCl, $NaHCO_3$, $KH_2PO_4$, $LiNO_3$, and $Na_2SO_4$, are injected into rabbits, of about the same weight, in solutions equivalent to 2.4 M NaCl, there is observed a marked decrease of approximately 70 per cent in the circulating mononuclear cells of the blood (Fig. 1). If the same salts are injected in solutions one-half and one-quarter this concentration, there is also a decrease in the number of the mononuclear cells, but only about half as great as is observed with the higher concentrations. Solutions of cane-sugar or glycerol equiosmotic with 2.4 M NaCl effect no significant change in the number of these cells. If only one injection of the salt in solution is made, the maximum decrease in the mononuclear cells is reached in about 3 hours, and 24 hours later the normal blood picture is observed. Since the results obtained are striking and uniform with all of the salts used, as long as the concentration of cations is equivalent, it is suggested that the changes observed are effected through the agency of the

cations. It is of interest to note that the cations employed belong to the same periodic group. Our experiments had to be discontinued

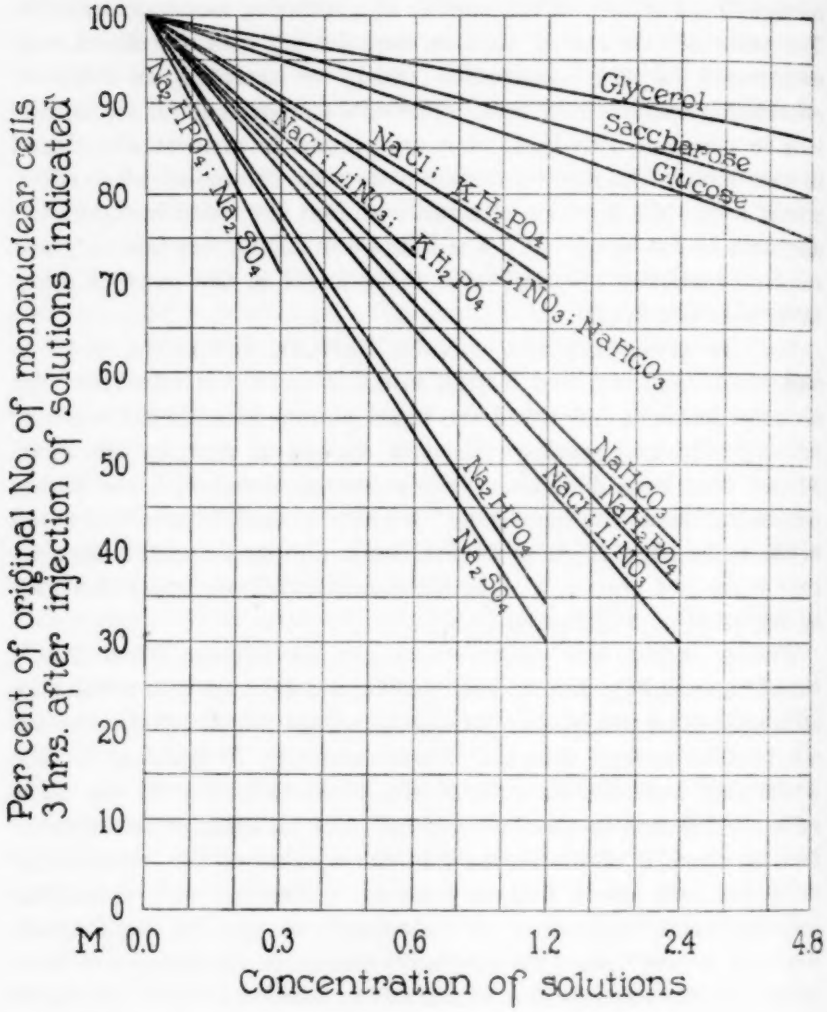


FIG. 1.

before it was possible to extend our observations to the effect of other cations and combinations of different cations, with reference to their antagonistic properties.

Our chief interest at present in the observations recorded centers about the analogy between the influence of the salts and the influence of x-radiations in so far as changes in the number of circulating mononuclear white blood cells is concerned. This analogy apparently increases in significance in the light of further observations made on animals where the injections of $NaHCO_3$ solutions were repeated several times. In these animals the decrease in the cells continues and the lymphoid tissue in the spleen shows evidence of definite injury analogous to the changes observed following x-ray exposure. The general health of the animal is not impaired in any observable manner. If this is true also in the case of repeated injections of other salts, as it would seem to be, it appears justifiable to assume that the changes are the result of a disturbance in the same chemical reaction. It does not follow, however, that the conditions leading to this disturbance are exactly the same. In fact the information we have relating to chemical changes brought about by x-rays indicates that these conditions are different. Our observations recall the experiences of Hertwig (2) who found that exposures of fertilized frogs' ova to radium not only retarded the developmental processes but that the larvæ hatched showed regular and characteristic changes. It was found later that the same retardation and subsequent changes could be induced by means of purely chemical agents. These results may be taken as further evidence of an end-result being affected by a disturbance in the same chemical reaction brought about through conditions entirely different. As a consequence of these experiences Hertwig stated that biological effects induced by radiation from radium were due to chemical reactions produced by them in protoplasm. This obviously is the case for both radium and x-rays.

## CONCLUSION.

A satisfactory correlation of our observations dealing with the influence of salts and those dealing with the influence of x-rays is not possible at present. Any far reaching conclusion is not permitted because the information we have at this time regarding the physical chemical conditions concerned in the process of injury, as well as that pertaining to the nature of radio-chemical reactions, is too meager. As far as the experiments with salts are concerned, it may be

said that we are dealing with ion effects, and their importance in physiological processes is made clear by the investigations of Loeb (3) and those of Osterhout (4). The results that we have obtained in our experiments present an interesting analogy between the effect of x-rays and certain salts on the lymphoid elements of the animal body. We regard this analogy as significant in that it presents suggestions regarding the chemical nature of x-ray effects in the animal body.

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# CONDUCTIVITY AS A MEASURE OF VITALITY AND DEATH.*

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The use of electrolytic conductance measurements in physiological research may be said to date back nearly a century. Eduard Weber at Halle in 1836[1] conceived that galvanic energy like other forms of energy might be useful in the human body, and that if this were the case there should be paths of differing conductivity through the tissues. His thorough and ingenious experiments command admiration, and his conclusions as to the epidermal locus of most of the electrolytic resistance of the body, and as to the effects upon it of warmth and moisture, are still valid. After Weber came du Bois-Reymond[2] who studied the resistance of muscle during contraction; Hermann[3] who showed that the resistance of muscle was greater across the fibers than along them; Ranke[4] who made careful studies of the decrease in resistance of plant and animal tissues upon death; and many others.

In 1897 there appeared almost simultaneously a number of studies of the resistance of red blood cells [5,6,7] which may be taken as the

* Approved for publication by the Surgeon General.

[1] Weber, E., Quaestiones physiologicae de phaenomenis galvano-magneticis in corpore humano observatis, Leipsic, 1836.

[2] du Bois-Reymond, E., Untersuchungen über thierische Elektricität, Berlin, 1849.

[3] Hermann, L., *Arch. ges. Physiol.*, 1872, v, 223.

[4] Ranke, J., Tetanus—eine physiologische Studie, Leipsic, 1865.

[5] Stewart, G. N., *J. Boston Soc. Med. Sc.*, 1896–97, i, No. 16, 18. This and subsequent papers are summarized by Stewart, G. N., *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 49.

[6] Róth, W., *Zentr. Physiol.*, 1897–98, xi, 271.

[7] Bugarszky, St., and Tangl, F., *Zentr. Physiol.*, 1897–98, xi, 297.

prototypes of studies on unicellular organisms, with which erythrocytes are in many ways comparable.

Recently a great impetus has been given to the use of the conductivity methods by the important generalizations which Osterhout[8,9,10] has been able to deduce from his studies on the marine alga *Laminaria*.

Conductance measurements have been made upon a large number of types of living material; but the results, especially when unicellular forms were used, have not been wholly satisfactory. The conductance of bacteria, for example, is oftentimes not an easy thing to determine, or to interpret. The present paper attempts to obviate a certain confusion which threatens to permeate the field by defining a set of concepts which seem best adapted to express the physiological state of the tissue, and by pointing out certain facts whose recognition is fundamental to further study of the subject. Some of these have been very briefly referred to in a previous paper.[11]

## I.

## *Definitions.*

To avoid needless repetition of qualifying phrases let us define two concepts. By "net conductance" we shall mean the difference in conductance of the current path between the electrodes when suspending fluid alone is present and when the same fluid is partially replaced by cells (or tissue), *expressed in per cent of the conductance in the former case*. This will ordinarily be negative in sign, and unless otherwise stated the negative sign will be understood. It is to be noted that as the conductivity of a tissue decreases, its "net conductance" as here defined increases. In the absence of tissue the conductance is that of a solution the same as that which bathes the tissue; in the presence of tissue both solution and tissue probably take part in the conduction of electricity.

Theoretical equations derived in much the same way as those previously published by the writer,[11] but assuming uniformly spaced as well as regularly arranged cells, show, however, that the conductance of some types of cells may be very small. The least

[8] Osterhout, W. J. V., *Science*, 1912, xxxv, 112.
[9] Osterhout, W. J. V., *Science*, 1914, xl, 488.
[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 145, 415, 611.
[11] Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1922, xix, 284.

conductive cells which the writer has measured were erythrocytes which in 24.2 per cent suspension gave a net conductance of 33.9 per cent. Applying the equations mentioned we may show that the resistivity of the cells was more than 11.3 times that of the solution; they would therefore conduct a very small part of the current, and changes in their resistivity would hardly affect the net conductance. The term "net resistance" has been used to express the difference in ohms found by subtracting "the resistance of the apparatus" from the resistance of the same apparatus containing *Laminaria*,[9] and represents in this case the actual resistance of the tissue in ohms. The net conductance as defined above will be shown to have the more general significance, and will be meant unless it is otherwise expressly stated.

The second concept which we wish to define is "dead conductance," by which we mean the net conductance of dead tissue in per cent of that of the same tissue in its normal living state; dead conductance is therefore 100 × (net conductance of dead tissue) ÷ (net conductance of living tissue).

## II.

## *Methods.*

In order to determine the net conductance of unicellular organisms it is necessary to know the conductance of the suspending fluid as well as that of the suspension. This may be done by filling the conductivity cell alternately with the suspension and with portions of suspending fluid obtained by centrifugation.[12,13,14] The same end is served by alternate centrifugation and resuspension of the organisms in an appropriate tube in which immersion electrodes may be placed in such a position as to lie wholly in the supernatant liquid after centrifugation; the resistance measurements are made immediately

[12] Green, R. G., Papers from the Mayo Foundation for Medical Education and Research and the Medical School, 1915–20, Philadelphia and London, 1921, 577. See also the paper by Green, R. G., and Larson, W. P., in *J. Infect. Dis.*, 1922, xxx, 550.

[13] Holland, D. M., personal communication.

[14] Shearer, C., *Proc. Cambridge Phil. Soc.*, 1916–19, xix, 263; *J. Hyg.*, 1919–20, xviii, 337.

before and immediately after resuspension. More concentrated suspensions may be used by the former method, but the latter involves less transfer of organisms from one vessel to another. It is also to be noted that during progressive changes in conductivity on the part of both cells and fluid, the observed conductance of the supernatant liquid, as found by the latter in contrast to the former method, is likely to be more nearly the same as the actual conductance of the fluid around the organisms at the moment when their conductance is being determined. The latter method is also more convenient, and has for all these reasons been selected in preference to the former method. In the writer's method two coaxial cylinders of bright platinum form the electrodes and are immersed in the suspension of cells. The tube containing the suspended cells may be removed, centrifugalized, and returned to its original position; the cells are thus brought to the bottom of the tube, and the electrodes lie wholly in the fluid which a moment before bathed the suspended cells. Successive readings may be made by alternate centrifugation and resuspension, and the supernatant fluid may be changed by decantation of the old and resuspension in the new fluid.

The electrical portion of the writer's apparatus is essentially a Wheatstone or, more properly, a Christie bridge circuit[15] in which one arm consists of the conductivity cell and another of variable resistance and capacitance in parallel. In some of the earlier experiments, one of which is described here, a variometer in series with the conductivity cell was used in place of the parallel capacitance in the adjacent arm. This parallel capacitance was substituted to simplify calculations not involved in this paper. This involves an error in apparent conductance which has been emphasized by McClendon,[16] and it therefore seems necessary to mention that this error is serious only in exceptional cases like that cited by McClendon; in the present experiments it is greatest in the experiments upon *Laminaria*, where it causes errors as great as several per cent. The results of these experiments are given in corrected form. In all the other experiments

[15] Christie, S. H., *Phil. Tr. Roy. Soc. London*, 1833, Part I, 95. Wheatstone called the attention of the public to Christie's paper, and his name has become associated with the bridge circuit there described.

[16] McClendon, J. F., *J. Biol. Chem.*, 1920, xliii, 317.

the error is less than that from other sources, *e.g.* about 0.6 per cent of the net conductance in the case of red blood cells, and the data are given in the uncorrected form.

It is probably always advisable to compensate for the shift of phase suffered by the current in the electrolytic cell by appropriate condensers or variometers. This affords a more definite end-point, although with large platinized electrodes good readings may be obtained without their use. Several methods of making such compensation are described by Taylor and Curtis,[17] Washburn,[18] and others. It is also essential to guard against changes in the electrode surfaces during an experiment. Platinized electrodes are notoriously difficult to keep clean, and it may often prove the safer course to use "blank" electrodes, especially in the presence of organisms like bacteria which continually set free into the solution complex organic substances, or which maintain unusual oxidation or reduction potentials.

Even blank electrodes are not free from errors of this sort. As an example an experiment upon the effect of mercuric chloride on a heavy suspension of *Bacillus butyricus* may be cited. Measurements were made upon the resistance of suspensions of bacteria in a 0.14 M solution of sodium chloride plus 0.1 per cent mercuric chloride, and as control, in a physiologically balanced solution having the same conductivity. In the former a gray coloration of the suspension was evident in about 1 hour and became steadily more pronounced, while at the same time the apparent resistance of both the suspending fluid and the suspension began to rise, and after about 7 hours became several times greater than at the beginning of the experiment. The exact extent of this rise could not be determined because the capacitance also increased, and ultimately exceeded the range of the available variometers used for compensation. Neither the gray color nor the rise in resistance was observed in the controls.

Electrodes previously used in the controls were then used in one of the suspensions containing mercuric chloride. The resistance measured with these fresh electrodes was nearly identical with the initial resistance. The affected electrodes were cleaned in boiling

[17] Taylor, W. A., and Curtis, H. L., *Phys. Rev.*, 1915, Ser. II, vi, 61.
[18] Washburn, E. W., *J. Am. Chem. Soc.*, 1916, xxxviii, 2431.

nitric acid for about 5 minutes, washed, and were then used for a resistance measurement. The readings were again identical with those of fresh electrodes.

Evidently reduction of mercuric chloride had occurred, presumably with deposition of a thin film of insoluble mercurous chloride upon the electrodes. A thin film of a relatively poor conductor would act like a leaky condensor and produce the observed phenomena, and it would be removed by the process of cleaning. The gray appearance of the suspension suggests that reduction was carried to the extent of depositing metallic mercury. Such difficulties as this are, of course, only occasional.

## III.

### EXPERIMENTAL.

Apparently the conductivity of cells in equilibrium with any non-injurious solution varies with that of the solution. If the conductivity of sea water were to be halved by dilution, the conductivity of a tissue immersed in it would also be approximately halved. In the case of organisms like bacteria this is peculiarly important since the conductivity of a new solution placed in contact with the organisms may be profoundly changed, and it would not be safe to assume that the conductivity of the bacteria was unaffected thereby. In fact the safest assumption seems to be that even in the case of tissue immersed in an injurious solution the conductivity of the tissue would vary as that of the surrounding solution if only the same degree of injury could be maintained long enough for equilibrium to be established.

That this equilibrium involves the conductance of the protoplasm itself appears probable, since it has been shown by Osterhout, using conductivity data,[19] and by the writer in his experiments on the free diffusion of salts through diaphragms of *Laminaria* tissue,[20] that the protoplasm, or at least some living substance forms a part of the pathway for the movement of ions in the tissue.

The following experiments upon several different organisms will serve to show how the principle operates.

[19] Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, iv, 1.
[20] Brooks, S. C., *Bot. Gaz.*, 1917, lxiv, 306.

A unicellular green alga *Chlorella* was grown in pure culture in Chodat-Grintzesco solution[21] plus 2 per cent saccharose for a period of 2 weeks, and the conductance of a dense suspension determined first in the nutrient solution, then in a similar solution concentrated to half its original volume by evaporation, and then again in the original nutrient solution (Table I). The small amount of available material renders the result less striking than it might be, since the net conductance here depends directly upon a small difference between

TABLE I.

*Effect of Conductance of the Surrounding Solution upon That of Chlorella.*

| Time. | | Solution. | Net conductance. | |
|---|---|---|---|---|
| *hrs.* | *min.* | | *per cent* | |
| 0 | 30 | Chodat-Grintzesco solution. | 4.8 | |
| 0 | 50 | | 4.7 | |
| 1 | 30 | | 4.3 | Average, 4.55 |
| 2 | 19 | | 4.3 | |
| 2 | 33 | | 4.7 | |
| 3 | 06 | | 4.5 | |
| 3 | 27 | Same; double strength. | 6.2 | |
| 3 | 46 | | 4.3 | |
| 4 | 04 | | 4.1 | Average, 4.27 |
| 4 | 20 | | 4.4 | |
| 4 | 44 | Same; original strength. | 0.3 | |
| 5 | 01 | | 3.6 | |

two relatively large quantities, and is therefore subject to unusually great error. In spite of this the net conductance approaches the same constant value in the different solutions. The first readings after each change of solution are seen to be affected by the previous condition; *e.g.*, when the change is to a more concentrated solution the apparent net conductance is high, since the protoplasm is still relatively too poor in electrolytes. The opposite variation occurs when the change is to a less conductive solution. (It is to be noted

[21] Chodat, R., and Grintzesco, I., Congrès International de Botanique à l'Exposition Universelle de 1900, Paris, 157–162.

that a high net conductance means not a highly conductive protoplasm but precisely the opposite.) The results are therefore just what would be predicted in the case of cells into and out of which salts diffuse rather slowly.

A somewhat more convincing proof was obtained when larger amounts of *Chlorella* were used. The material was raised in the same way, but Perrier's artificial sea water[22] diluted to one-eighth its original concentration was used as the more dilute, and double this strength (*i.e.* one-fourth strength) Perrier's solution as the more concentrated medium. The normal net conductance of *Chlorella*

TABLE II.

*Effect of Conductance of the Surrounding Solution upon That of Chlorella.*

| Time. | | Solution. | Net conductance. | |
|---|---|---|---|---|
| | | | Observed. | Mean. |
| *hrs.* | *min.* | | *per cent* | *per cent* |
| 2 | 40 | Perrier's sea water; $\frac{1}{8}$ strength. | 28.8 | |
| 3 | 23 | | 28.7 | 28.9 |
| 4 | 00 | | 29.1 | |
| 5 | 10 | Same; $\frac{1}{4}$ strength. | 28.0 | |
| 5 | 50 | | 27.8 | 27.2 |
| 6 | 27 | | 27.0 | |
| 7 | 07 | | 25.8 | |

is maintained for at least 24 hours in either of these solutions. Table II summarizes the experiment. Upon transfer to the stronger solution the cells shrank slightly, and since the net conductance is proportional to the volume of cells over short ranges in suspensions of the density used,[11] it is possible to correct for the change in volume. The corrected figure should tell us more nearly the conductance of the cell material itself. It is found to be 28.3 per cent, a figure which agrees well with the original figure of 28.9 per cent.

Table III shows that the behavior of *Bacillus butyricus* is similar. A 24 hour culture on glycerol agar was used. Again the net conductance in the two solutions coincides within the limits of error of the method.

[22] Perrier, E., *Compt. rend. Acad.*, 1890, cx, 1076.

The experiments were also extended to include *Laminaria aghardii*, which was studied in a manner essentially like that described by Osterhout,[8] the thalli being cut into discs and these piled like a roll of coins into a closely fitting glass tube. At each end of this tube was a paraffin block in which was a well open at the top and connecting with the lumen of the tube. Platinized platinum electrodes 1 cm. in diameter were inserted into the wells and held by means of rubber stoppers. Variation in the distance between the electrodes was not great enough to disturb the experiments. The current passed from one electrode through the glass tube to the other. The tube

TABLE III.

*Effect of Conductance of the Surrounding Solution upon That of B. butyricus.*

| Time. | | Solution. | Net conductance. | | Net conductance corrected for change of cell volume. |
|---|---|---|---|---|---|
| | | | Observed. | Mean. | |
| *hrs.* | *min.* | | *per cent* | *per cent* | *per cent* |
| 0 | 00 | Perrier's sea water; $\frac{1}{8}$ strength. | 16.0 | | |
| 1 | 01 | | 17.5 | 16.8 | 16.8 |
| 2 | 21 | | 16.6 | | |
| 3 | 24 | | 17.2 | | |
| 5 | 12 | Same; $\frac{1}{4}$ strength. | 14.6 | | |
| 6 | 05 | | 15.4 | 15.0 | 17.2 |
| 7 | 09 | | 14.9 | | |

was first filled with sea water and the conductance determined; then the discs of *Laminaria* tissue were inserted and another determination was made. After this the tissue was placed in half strength sea water (equal parts of distilled and sea water) for about one-half hour, and then the conductance in the half strength sea water determined as before. The experimental protocol (Table IV) not only serves to confirm the results of the previous experiments, but also furnishes a striking example of the fact, which also exists in the other cases cited, that the net resistance in ohms may be a very misleading quantity. The same remark applies to the net conductance in reciprocal ohms as opposed to the net conductance in per cent used by the writer. It is obvious that the vitality or injury of any tissue is

best expressed in terms of net conductance (or net resistance) in per cent. In comparing two lots of tissue allowance must, of course, be made for the relative volumes of tissue and solution present, but for comparative purposes, the sequence of changes in any one lot of tissue may be followed by taking the normal net conductance as a basis to which all other figures are referred.

Another point which seems to need emphasis, is that dead plant or animal cells of all the kinds which I have studied, and under all conditions which I have been able to create with one exception which will be discussed in a subsequent paper, offer more or less resistance

TABLE IV.

*Effect of Conductance of the Surrounding Solution upon That of Laminaria aghardii.*

| Time. | | Solution. | Gross resistance. | | Change of resistance due to presence of tissue. | Net conductance. |
|---|---|---|---|---|---|---|
| | | | Solution only. | Solution plus tissue. | | |
| *hrs.* | *min.* | | *ohms* | *ohms* | *ohms* | *per cent* |
| 0 | 20 | Sea water. | 148.5 | 933.7 | 785.2 | 84.1 |
| 1 | 03 | Same; ½ strength. | 289.6 | 1,788.0 | 1,498.4 | 83.3 |
| 2 | 02 | Same; full strength. | 151.2 | 949.7 | 798.5 | 84.1 |

to the passage of an electrical current. This is of particular interest in view of an impression which appears to have arisen, that dead tissue ordinarily has the same electrical resistance as the surrounding fluid. Such an impression may possibly have had its origin in too literal an interpretation of Osterhout's statement[8] regarding *Laminaria* that "In all cases the resistance after killing fell to *about*"[23] that of a similar cylinder of sea water. As a matter of fact, the resistance of suspensions of some unicellular forms hardly changes when they are killed. Under some conditions the resistance may be increased by the killing process.[2,3] It is necessary to select carefully the means used to kill the organism. Dense bacterial suspensions, for example, may survive doses enormously exceeding the traditional fatal concentrations; nor can one confidently say that any certain

[23] The italics are mine.

degree of heating is satisfactory since too much heat may raise the resistance of some forms rather than lower it.

For example, of two similar portions of a suspension in one-fifth strength unbuffered Ringer-Tyrode solution of *Bacillus coli* (20 hour culture on trypagar), one was kept at 62°C. for 5 minutes, while the other was left at 38.2°C., the temperature at which the readings were made. Subsequent measurements showed that the net conductance of the heated specimen even when decreased by correcting for a slight increase in volume was still 10.7 per cent as compared with 8.8 per cent for the control. Plate cultures showed that the bacteria in the heated portion were essentially all dead, only 57 colonies developing, whereas innumerable colonies appeared in the control. The exact reason for such a change is not apparent; but since the conductivity of the suspending fluid increased, just as found by Green,[12] electrolytes must be diffusing from the cell, and imperfect establishment of equilibrium would have decreased rather than increased the net conductance. This then is not the explanation.

It may be suggested that such a rise in net conductance is due merely to minute air bubbles developing in the solution. But the following experiment shows that similar effects may be produced by other agents, which would not cause the formation of air bubbles. A suspension of *Bacillus coli* of pH 7.7 was divided into four portions, of which two were reserved as controls. Before treatment the net conductance of the control tubes was 3.25 per cent, of the others 4.10 per cent. After 2 hours and 40 minutes in 0.015 M HCl the pH of the treated tubes was 3.4; that of the controls still 7.7. Plate cultures showed the controls to be increasing in numbers, while no colonies developed from the acidulated suspensions; yet the latter had a net conductance (8.15 per cent) more than double that of the controls (3.95 per cent). Still other examples will be found in Table V. An increase in the resistivity of the cells appears to be the only plausible explanation of such an increase of the net conductance.

Still other methods of killing were tried. Organic solvents and heavy metal salts were avoided because of the probability that they would extract fat-soluble substances from, or otherwise profoundly affect non-living material. Liquid air, affording a temperature of

about −190°C. seemed to offer an ideal means of killing.[24] Two portions of a suspension of *Bacillus coli* were both exposed to liquid air for a period of 15 minutes; the tubes were smartly struck several times in the meanwhile to promote formation of ice crystals, then

TABLE V.

*"Dead Conductance" of Various Types of Cells.*

| Organism. | Killing agent. | Duration of action. | Dead conductance. | | Notes. |
|---|---|---|---|---|---|
| | | | Observed. | Mean. | |
| | | *min.* | *per cent* | *per cent* | |
| *B. butyricus.* | 65–72°C. | 65 | 54.7 | | |
| Same suspension. | 65–80° " | 105 additional. | 70.7 | | |
| *Saccharomyces cerevisiæ.* | 100° " | 5 | 65.0 | | |
| *Chlorella* sp. | 64–68° " | 48 | 71.0 | | |
| " | 75–80° " | 60 | 109.7 | | |
| " | 55–63° " | 75 | 92.3 | 89.2 | |
| " | 55–61° " | 46 | 90.8 | | |
| " | 72–81° " | 42 | 82.0 | | |
| " | Tricresol, 6.7 per cent. | 35 | 136.9 | | |
| | | 1,320 additional. | 203.8 | | |
| " | −190°C.± | 20 | 95.0 | | (Liquid air.) |
| " | −190° " ± | 45 | 91.0 | | Not all dead. |
| Rabbit erythrocytes. | 73–75° " | 15 | 45.8 | | |
| " " | 70–72° " | 30 | 46.6 | 45.8 | |
| " " | 68–76° " | 30 | 45.0 | | |
| " " | 63–67° " | 30 | 46.0 | | |

thawed, and were again given a 15 minute exposure to liquid air, after which they were brought to a temperature of 38°C. The immediate results were most gratifying. The net conductance fell

[24] The liquid air used in these experiments was most generously supplied by the Cryogenic Laboratory of the Bureau of Mines, United States Department of the Interior.

from 16.3 per cent immediately before exposure to 9.5 per cent immediately afterwards. This, by far the largest drop in net conductance observed up to that time, seemed to be the clew to the real "dead conductance." Unfortunately, plates made before and after exposure to liquid air showed more numerous colonies after the treatment than before.[25] Repetition of the experiment served but to confirm the disconcerting fact that the net conductance fell, but the bacteria lived and throve.

Recourse was again had to heating as a means of killing and evidence obtained that the net conductance of *Bacillus coli* could be lowered by heat. In one experiment, for example, the temperature rose slowly from 80–94°C. during 30 minutes. The bacteria were then washed several times in fresh suspending solution and plate cultures made. No colonies developed. The net conductance fell from an average of 4.15 per cent for the 4 preceding determinations to 2.10 per cent for the following two, after which the experiment was terminated. The dead conductance was accordingly 50.6 per cent.

Other organisms gave somewhat similar results which are condensed for the sake of brevity in Table V.

It will be seen that all these tissues retain some net conductance in spite of all attempts to find a means of killing which would leave the cells with none. It seemed desirable to make comparisons between the writer's method, and that of Osterhout to eliminate the possibility that there might be a difference in the results due to some systematic error. If both methods were sound, experiments upon *Laminaria* by the writer's method should agree with those of Osterhout[8] and in particular the dead conductance of killed *Laminaria* should be very small or entirely absent.

Accordingly, fresh healthy *Laminaria* thallus was cut into pieces about 1 mm. square, and the resistance of a suspension of such squares compared with that of the surrounding sea water. Since the small pieces settled completely to the bottom of the tube in 2 or 3 minutes, it was only necessary to stir up the suspension and make two determinations of the resistance, one immediately and one in about 5 minutes.

[25] Only one dilution was made and the colonies were so abundant as to defy more exact enumeration.

In the first experiment the normal net conductance was found to be 38.8 per cent, and after heating to 46–51°C. for 20 minutes it was 9.5 per cent. The "dead conductance" was therefore 24.5 per cent, whereas the tissue appeared to be dead. Repetition of the experiment lead to no fundamental change in this result. Since it seemed possible that fine cutting might have injured the tissue and thus lowered the assumed normal net conductance, experiments were tried in which a piece of thallus about 5 × 25 cm. was rolled up into a cylinder fitting tightly into the tube containing sea water, and leaving a space in the center just large enough to admit the electrodes and their supporting rod. The current was thus forced to pass chiefly through the tissues.

TABLE VI.

*"Dead Conductance" of Laminaria Tissue According to the Writer's Method.*

| Killing agent. | Duration of action. | | Dead conductance. |
|---|---|---|---|
| | *hrs.* | *min.* | *per cent* |
| Formaldehyde, 1 per cent | 23 | 20 | 51.0 |
| " 2 " " | 24 | 10 | 44.6 |
| Ethyl alcohol, 25 per cent | 1 | 40 | 23.4 |
| Heat 46–51°C | 0 | 20 | 24.5 |
| " 45–50°C | 2 | 35 | 26.9 |

Tested in this manner a strip of thallus had a net conductance of 52.3 per cent before exposure for 135 minutes to a temperature of 45–50°C., and of 14.1 per cent after exposure; the dead conductance was therefore 26.9 per cent. The other killing agents tried and the results obtained are given in Table VI. These experiments were carried on in half strength, or in the last case in $\frac{3}{4}$ strength sea water. The net conductance in sea water at such dilutions remained normal for more than the duration of the experiments. In all cases the "killed" tissues were green and flaccid long before the close of the experiment, thus giving every visible evidence of being dead. The experiments with formaldehyde disagree with the others which in general suggest a characteristic dead conductance of about 25 per cent.

Since this figure was considerably in excess of the dead conductance expected, an apparatus essentially like that used by Osterhout was set up. This has been described above. Various killing agents were again tried, and the results agreed substantially with those found by the writer's method. They are summarized in Table VII. These experiments were all done in full strength sea water. Fig. 1 shows that during the progress of heating the net conductance approached a constant value which may be considered as indicative of death.

In the experiments on *Laminaria*, it will be noticed that the "dead conductance" is in all cases practically identical for all the experiments in which portions of the same lot of discs were used, irrespective

TABLE VII.

*"Dead Conductance" of Laminaria Tissue According to Osterhout's Method.*

| Killing agent. | Duration of action. | | Dead conductance. | |
|---|---|---|---|---|
| | *hrs.* | *min.* | *per cent* | |
| Formaldehyde, 4 per cent..... | 4 | 0 | 64.5 | Same tissue. |
| " 4 " " ..... | 13 | 10 | 64.5 | |
| Heat 45°C..... | 1 | 0 | 35.0 | All from same original thalli. |
| " 37–39°C..... | 2 | 0 | 36.5 | |
| Ethyl alcohol, 25 per cent..... | 0 | 25 | 37.0 | |
| Heat 36–39°C..... | 4 | 29 | 21.7 | From same thalli. |
| " 36–39°C..... | 7 | 50 | 21.9 | |

of the manner of killing. In these experiments the dead conductance is probably determined by the state of the original tissue, which may have been in some cases in rather poor condition, rather than by the manner of killing. This would lead us to expect that under the most favorable conditions, *i.e.* with the original material having the maximum normal net conductance, the dead conductance might be as low as 10 or 20 per cent.

It will also be noted that in the case of *Bacillus coli* the existence of a considerable dead conductance is in agreement with the independent findings of other investigators in this country,[12],[13] but apparently in direct conflict with those of Shearer.[14] Certain of the writer's experiments on erythrocytes suggest that the conflict is only apparent,

and may easily be explained as a result of differing experimental conditions. Discussion of this point will be taken up in another paper.

It will be apparent from these data that the writer's method and that used by Osterhout agree in assigning to all the organisms and cells studied a certain net conductance, or resistance to the passage of current even when they are presumably dead. This dead conductance is least in *Laminaria* and is greater in other forms in the approximate order: blood cells, yeast, *Bacillus butyricus*, *Chlorella*,

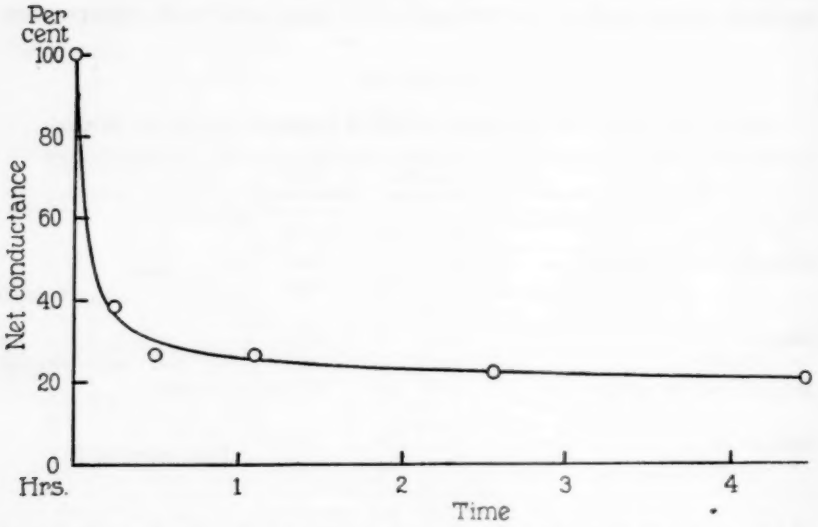


FIG. 1. The progressive decrease in the net conductance of *Laminaria* in sea water at 36–39°C. The measurements were made at room temperature.

*Bacillus coli*. As has already been pointed out in the case of all except *Laminaria* the dead conductance is relatively greater in those cells normally exposed to a fluctuating environment.[11] This correlation suggests the possibility that cell walls (or at least some non-living structure) might help to protect the protoplasm of such cells from injury due to abrupt changes in the electrolyte concentration, by impeding diffusion of the ions of electrolytes into and out of the protoplasm. This correlation may be only apparent, since similar effects might be produced by other than external structures, by the

presence of a certain proportion of dead cells in the population assumed to give the normal net conductance, or other causes which will suggest themselves to the reader. It is hoped that further studies will elucidate this point.

In order to forestall possible misconceptions it may be well to point out that the fact that *Laminaria* has, when dead, a resistance higher than that of sea water, in no wise affects the theory of injury and recovery based by Osterhout[10] upon calculations from data upon *Laminaria*. These calculations do not depend on the resistance of sea water, but allowance is made for the observed resistance of killed tissue.

## IV.

## SUMMARY.

The conductance of *Laminaria*, *Saccharomyces*, *Bacillus coli* and *Bacillus butyricus*, *Chlorella*, and of red blood cells has been studied by the writer's method, and *Laminaria* by that of Osterhout. For the material studied it has been found that:

1. The conductance of living tissue is closely proportionate to, and determined by that of the surrounding fluid with which it is apparently in equilibrium. Changes in the conductance of the fluid are quickly followed by compensatory changes in that of the tissue.

2. A quantity is defined which is independent of the conductivity of the fluid bathing the tissues. This is called the "net conductance."

3. All the tissues studied, even when dead, offer a resistance to the passage of current greater than that of the surrounding solution. Exceptions which occur under certain conditions will be discussed in a later paper.

4. In view of the wide variety of material studied it seems admissible, in the absence of any evidence to the contrary, to suppose that these conclusions are generally applicable.

A grant from the Elizabeth Thompson Science Fund was used by the writer previous to the assumption of his duties at the Hygienic Laboratory in the development of the method used in these studies, and is hereby gratefully acknowledged, as is also the courtesy of the Marine Biological Laboratory at Woods Hole, Massachusetts, where portions of the work were carried on.

# THE IONIZATION OF PROTEIN CHLORIDES.

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## I.

## INTRODUCTION.

When isoelectric protein is dissolved in dilute hydrochloric acid, the concentration of hydrogen ion in the hydrochloric acid is diminished. This fact has led to the belief that the acid combines chemically with the protein, the reaction being similar to the combination of acid with an amino-acid or any other amphoteric electrolyte. If this idea is correct it should follow that the protein chloride so formed should itself be ionized, yielding a positive ion composed of protein with $H^+$ added, the corresponding negative ion being chloride ion. The degree of ionization of the protein chloride should be of the same order as that of ammonium chloride; that is, protein chloride should be a highly ionized salt. Accordingly it would be expected that the concentration of chloride ion in hydrochloric acid should be diminished very little, if at all, by the addition of protein.

This idea was tested by Loeb,[1] who found that the chloride ion concentration of hydrochloric acid, as measured by the calomel electrode, was not appreciably altered by the presence of 1 per cent gelatin. Similar results had been obtained with gelatin by Manabe and Matula,[2] who found, however, that in the case of dialyzed ox serum and serum albumin there appeared to be considerable combination of chloride ion with the protein. Robertson[3] has proposed a theory of protein ionization which is based on the idea that compounds

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 42.

[2] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

[3] Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 167.

of protein with acid or alkali do not yield on electrolytic dissociation the simple anion of the acid or cation of the base in question. In order to determine which of these views might be of general application, measurements have been made of the chloride ion concentration in solutions of the chlorides of several proteins.

## II.

## EXPERIMENTAL.

The proteins used were gelatin, egg albumin, casein, edestin, and serum globulin. The gelatin was rendered isoelectric as described by Loeb.[4] The egg albumin was purified by the method of Sörensen.[5] The casein was prepared from milk by the method of Van Slyke and Baker,[6] washed free from HCl, and dried with acetone. The edestin was prepared from ground hemp-seed by the method of Osborne,[7] and the serum globulin was obtained from ox serum by the procedure given by Robertson.[8] The edestin and serum globulin were not dried, but were washed free from salt by decantation with water of a pH close to that of their isoelectric points. With each protein a series of solutions was made up so that 100 cc. contained in each case 1 gm. of protein, as determined by dry weight determinations. Each solution contained also hydrochloric acid, varying in concentration from 0.001 to 0.100 molar.

The chloride ion concentrations were determined by means of the silver-silver chloride electrode. The electrodes were prepared as described by MacInnes and Parker,[9] except that a short spiral of platinum wire was used instead of platinum gauze, being plated electrolytically first with silver and then with silver chloride. These electrodes were fitted into vessels of the type devised by Clark for hydrogen electrode work, as modified by Cullen[10] by the introduction

[4] Loeb, J.,[1] p. 35; *J. Gen. Physiol.*, 1918–19, i, 39; *J. Am. Chem. Soc.*, 1922, xliv, 213.

[5] Sörensen, S. P. L., and Höyrup, M., *Compt. rend. trav. Lab. Carlsberg*, Copenhagen, 1915–17, xii, 12; *Z. physiol. Chem.*, 1918, ciii, 16.

[6] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

[7] Osborne, T. B., in Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin, 1910, ii, 289.

[8] Robertson, T. B.,[3] p. 40.

[9] MacInnes, D. A., and Parker, K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1445.

[10] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 521.

of a thermometer. The vessels were connected by a salt bridge of saturated potassium chloride to a saturated potassium chloride calomel cell. It was at first thought that reliable results could not be secured in this way owing to possible diffusion of KCl into the cell while the stop-cock was opened to make a reading, and to avoid this some measurements were carried out with a salt bridge of saturated ammonium nitrate. These measurements gave higher values for pCl ($pCl = -\log [Cl^-]$) than were obtained with the KCl bridge. That this difference was not due to diffusion of KCl into the cell is indicated by the fact that the pCl values for HCl solutions agree closely with the pH values. With $NH_4NO_3$ and HCl alone, equally good readings were obtained. The use of $NH_4NO_3$ was abandoned in favor of KCl because it was found that the pCl values obtained with gelatin chloride did not check those of Manabe and Matula[2] and of Loeb,[1] but were higher, as well as less closely reproducible than the results obtained with KCl. Apparently this difference is due to a contact potential difference at the junction of the $NH_4NO_3$ solution and the protein chloride solution, which does not exist at the junction with saturated KCl. The apparatus was kept in an air bath at $25° \pm 0.2°$, the temperature being checked by the thermometer in each cell.

The measurements of E. M. F. were made with a Leeds and Northrup portable potentiometer, reading to 0.5 millivolt. The sensitivity of this instrument was found to be ample in solutions containing more than 0.002 N HCl. It was found possible to bring the solutions to equilibrium with the electrodes by the same procedure as that used in hydrogen electrode work. Each cell, with its electrode in place, was washed with the solution, half filled, and rocked for 3 minutes. A reading was taken, and the cell was emptied, refilled with the same solution, and rocked again. This was repeated until the readings obtained with two successive fillings of the cell agreed to within 0.5 millivolt, which was usually found to be the case on the second and third fillings of the cell. That this procedure gave the true equilibrium values for the E. M. F. is borne out by the agreement of the values for pCl and pH obtained with HCl solutions alone. This was confirmed by some preliminary measurements in which the electrodes were allowed to stand over night in the solutions before readings were taken. The pCl values obtained in this way with pure HCl solutions

and with gelatin chloride solutions were nearly the same as those obtained by the more rapid procedure.

Another conceivable source of error might be a change in the solubility of AgCl due to the presence of the protein. Measurements of pCl in solutions containing only protein and water did indicate a somewhat greater concentration of chloride ion than that calculated from the known solubility of AgCl in water. A correction could be made for this, as was done by Oryng and Pauli[11] in the case of calomel, by subtracting the chloride ion concentration so observed from that observed with protein chloride and water. When this was done, however, it was found that the correction was negligible except in the two most dilute solutions (0.001 and 0.002 N HCl). Since with these solutions the readings were not very reliable, and since in all other cases the correction amounted to less than 0.01 pCl, the use of the correction was considered unnecessary.

The determinations of hydrogen ion concentration were made with the same apparatus, except that platinized platinum electrodes were inserted in place of the silver chloride electrodes. The procedure was identical except that a stream of hydrogen was passed through the empty cell for 10 seconds just before the solution was put in.

The values for pH and pCl are based on 0.100 M HCl as a standard, its pH or pCl being taken as 1.035 at 25°. The electrodes were standardized against this HCl before and after each day's use. Accordingly, the values for pH and pCl were calculated by the use of the Nernst formula in the form

$$\text{pH (or pCl)} = \frac{E_x - E_{HCl}}{0.0591} + 1.035$$

where $E_x$ and $E_{HCl}$ represent the E. M. F. in volts observed with the solution under investigation and the 0.100 M HCl respectively.

## III.

## DISCUSSION.

The values for pCl obtained with pure HCl and with the protein chloride solutions are given in Table I. The reliability of the method is indicated by the agreement between the values for pH and pCl in the case of HCl free from protein. In the case of gelatin and egg

[11] Oryng, T., and Pauli, W., *Biochem. Z.*, 1915, lxx, 368.

albumin the pCl values are nearly the same as those for pure HCl. This indicates that the chlorides of these proteins are practically completely ionized in the solutions studied. With casein, edestin, and serum globulin the values are somewhat higher, indicating that the ionization of the chlorides of these proteins is not quite complete.

In order to compare the concentration of ionized protein chloride with the total concentration of protein chloride formed in any given

TABLE I.

*Measurements of Chloride Ion Concentration in Protein Chloride Solutions.*

| Concentration of HCl. | −log HCl. | pH of HCl. | pCl of HCl. | pCl of 1 per cent protein chloride solutions. | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Gelatin. | Egg albumin. | Casein. | Edestin. | Serum globulin. |
| N | | | | | | | | |
| 0.001 | 3.00 | 3.00 | 2.97 | (2.90)* | (2.88) | — | (2.89) | (2.93) |
| 0.002 | 2.70 | 2.70 | 2.69 | (2.67) | (2.63) | — | (2.64) | (2.70) |
| 0.003 | 2.52 | 2.52 | 2.51 | 2.52 | 2.52 | — | 2.51 | 2.58 |
| 0.004 | 2.40 | 2.40 | 2.41 | 2.41 | 2.41 | — | 2.36 | 2.45 |
| 0.005 | 2.30 | 2.30 | 2.28 | 2.31 | 2.32 | 2.36 | 2.29 | 2.35 |
| 0.006 | 2.22 | 2.22 | 2.20 | 2.24 | 2.25 | 2.25 | 2.24 | 2.27 |
| 0.007 | 2.15 | 2.15 | 2.15 | 2.18 | 2.19 | 2.19 | 2.19 | 2.23 |
| 0.008 | 2.10 | 2.10 | 2.10 | 2.12 | 2.09 | 2.16 | 2.12 | 2.15 |
| 0.010 | 2.00 | 2.00 | 1.98 | 2.02 | 2.00 | 2.04 | 2.05 | 2.05 |
| 0.015 | 1.82 | 1.83 | 1.79 | 1.85 | 1.85 | 1.86 | 1.84 | 1.89 |
| 0.020 | 1.70 | 1.71 | 1.68 | 1.73 | 1.73 | 1.74 | 1.75 | 1.79 |
| 0.030 | 1.52 | 1.54 | 1.52 | 1.54 | 1.56 | 1.57 | 1.54 | 1.58 |
| 0.040 | 1.40 | 1.42 | 1.40 | 1.42 | 1.42 | 1.44 | 1.40 | 1.46 |
| 0.050 | 1.30 | 1.33 | 1.31 | 1.33 | 1.33 | — | 1.34 | 1.37 |
| 0.100 | 1.00 | 1.035 | 1.035 | 1.08 | 1.05 | — | 1.06 | 1.12 |

* Values in parentheses were uncertain on account of the low sensitivity of the galvanometer used.

solution, the amounts of combined hydrogen ion were determined. This was done by means of measurements of pH of the protein chloride solutions and of pure HCl solutions. A large scale graph was made of the pH of HCl solutions as a function of the concentration of HCl. From this curve were read off the amounts of free HCl corresponding to the pH actually measured in each protein chloride solution. These amounts of free HCl were subtracted from the total HCl present in each protein chloride solution, the difference representing the $H^+$ combined with the protein or the total equivalent concentration of

protein chloride formed. Table II gives the pH values and the amounts of combined hydrogen ion.

The equivalent concentration of ionized protein chloride was obtained by taking the difference between the measured concentrations of $Cl^-$ and $H^+$ in each solution. The validity of this method of calculation follows from the fact that the solution must be electrically neutral. Representing protein by P, the possible equilibria in the solution may be indicated as follows:

$$P + H_2O \rightleftarrows HPOH \rightleftarrows HP^+ + OH^-$$
$$HCl \rightleftarrows Cl^- + H^+$$
$$\downarrow\uparrow \quad \downarrow\uparrow$$
$$HPCl \quad H_2O$$

TABLE II.

*Total and Ionized Protein Chloride, Concentrations in Milli-Equivalents per Liter.*

| Concentration of HCl. | Gelatin chloride. | | | Egg albumin chloride. | | | Casein chloride. | | | Edestin chloride. | | | Serum globulin chloride. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH | Total. | Ionized. | pH | Total. | Ionized. | pH | Total. | Ionized. | pH | Total. | Ionized. | pH | Total. | Ionized. |
| 0 | 4.80 | 0 | 0 | 5.63 | — | — | | | | 5.75 | 0 | 0 | 6.15 | 0 | 0 |
| 1 | 4.44 | 1.0 | 1.2 | 4.98 | — | — | | | | 4.78 | 1.0 | 1.3 | 4.73 | 1.0 | 1.2 |
| 2 | 4.25 | 2.0 | 2.1 | 4.47 | 0.7 | 1.0 | * | | | 4.62 | 2.0 | 2.3 | 4.16 | 1.9 | 1.9 |
| 3 | 4.00 | 2.9 | 2.9 | 4.02 | 1.6 | 1.6 | | | | 4.36 | 3.0 | 3.1 | 3.87 | 2.8 | 2.5 |
| 4 | 3.80 | 3.8 | 3.7 | 3.60 | 2.5 | 2.3 | | | | 4.15 | 3.9 | 4.3 | 3.61 | 3.8 | 3.2 |
| 5 | 3.64 | 4.8 | 4.7 | 3.35 | 3.3 | 3.0 | 3.07 | 4.2 | 3.5 | 3.97 | 4.9 | 5.0 | 3.36 | 4.6 | 4.1 |
| 6 | 3.43 | 5.6 | 5.4 | 3.15 | 4.0 | 3.6 | 2.94 | 4.9 | 4.5 | 3.81 | 5.8 | 5.6 | 3.25 | 5.4 | 4.8 |
| 7 | 3.25 | 6.4 | 6.1 | 2.97 | 4.6 | 4.0 | 2.79 | 5.4 | 4.9 | 3.61 | 6.8 | 6.3 | 3.00 | 6.0 | 4.9 |
| 8 | 3.09 | 7.2 | 6.8 | 2.83 | 5.2 | 5.3 | 2.67 | 5.8 | 4.8 | 3.50 | 7.7 | 7.3 | 2.81 | 6.4 | 5.6 |
| 10 | 2.73 | 8.1 | 7.7 | 2.60 | 6.1 | 6.1 | 2.42 | 6.2 | 5.3 | 3.21 | 9.4 | 8.3 | 2.63 | 7.6 | 6.6 |
| 15 | 2.19 | 8.6 | 7.6 | 2.21 | 7.5 | 6.6 | 2.08 | 6.7 | 5.5 | 2.63 | 12.6 | 12.2 | 2.20 | 8.7 | 6.6 |
| 20 | 1.97 | 9.2 | 7.9 | 1.99 | 8.5 | 7.1 | 1.87 | 6.3 | 4.7 | 2.19 | 13.6 | 11.3 | 1.91 | 7.6 | 3.9 |
| 30 | 1.68 | 8.5 | 7.9 | 1.72 | 9.2 | 7.1 | 1.63 | 5.8 | 3.5 | 1.81 | 14.2 | 13.3 | 1.70 | 9.6 | 6.3 |
| 40 | 1.52 | 8.4 | 7.8 | 1.54 | 8.7 | 7.9 | 1.48 | 5.4 | 3.2 | 1.59 | 13.3 | 14.1 | 1.52 | 8.5 | 4.5 |
| 50 | 1.41 | 8.9 | 7.9 | 1.42 | 8.7 | 7.5 | | | | 1.47 | 14.6 | 11.8 | 1.41 | 9.0 | 3.8 |
| 100 | 1.08 | 10.1 | 0 | 1.08 | 8.7 | 4.6 | | | | 1.09 | 2.01 | 5.8 | 1.06 | 5.9 | −11.2 |

* 1 gm. casein is completely soluble in 100 cc. of HCl only between 0.005 and 0.040 N.

The acidic ionization $HPOH \rightleftharpoons H^+ + POH^-$ may safely be neglected in acid solutions; therefore $[HP^+] + [H^+] = [Cl^-] + [OH^-]$. In acid solutions $[OH^-]$ is negligible in comparison with the other terms; therefore $[HP^+] = [Cl^-] - [H^+]$. If the protein acts as a poly-acid base the same reasoning applies if concentrations are expressed in equivalents. The equivalent concentrations of ionized protein so obtained are given in Table II.

The order of the degree of ionization of the protein chlorides is shown by the curves in Figs. 1 to 5, in which the equivalent concentrations

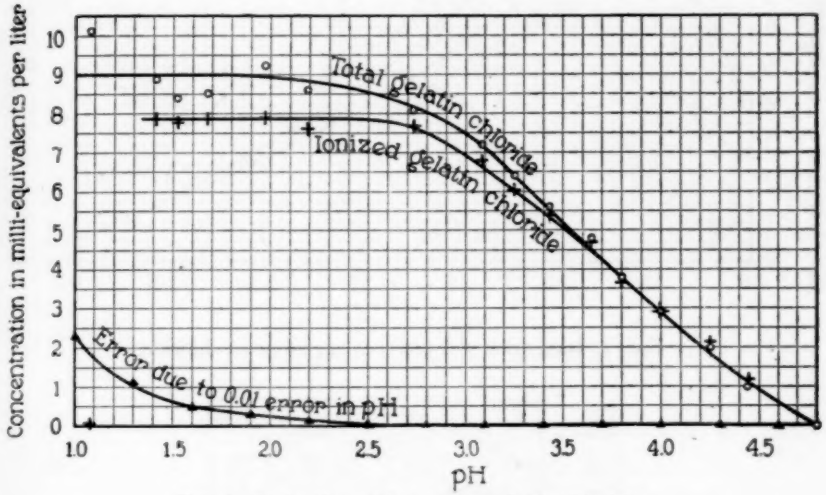


FIG. 1. Ionization of 1 per cent gelatin chloride.

of protein chloride found (or $[H^+]$ combined) and of ionized protein chloride (or $[Cl^-] - [H^+]$) are plotted as functions of the pH. The ratio of the ordinates of the two curves in each figure, at the same pH, indicates the fraction of protein chloride ionized. It is evident that this ratio is close to 100 per cent except in the most acid solutions. Only in the most acid solutions is there some indication of a repression of the ionization of the protein chloride by the high concentration of $Cl^-$ from the HCl added.

The irregularity of the results in the more acid solutions is partly explained by the lower curve in Fig. 1, which represents the error in concentration due to an error of 0.01 in pH. When it is considered

that each of the upper curves represents the difference between two concentrations, and that there may be an error of 0.02 in the values for pH or pCl, the scattering of the points is not surprising.

The curve for the HCl combined with gelatin agrees closely with that previously found[12] at 33°, and the values for the pH of casein solutions

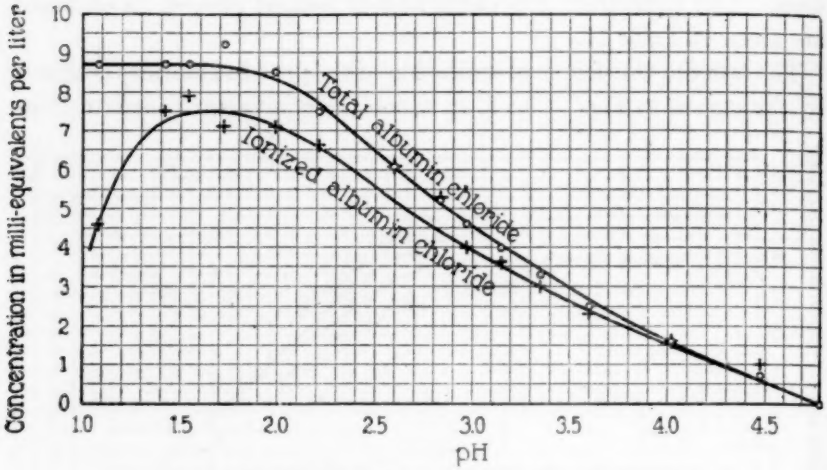


FIG. 2. Ionization of 1 per cent egg albumin chloride.

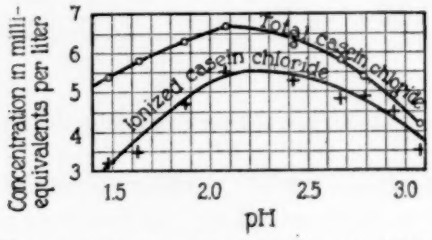


FIG. 3. Ionization of 1 per cent casein chloride.

agree with those given by Loeb.[13] The pH values of the egg albumin solutions agree fairly well with Loeb's values[14] if correction is made for the HCl required (namely, $1.35 \times 10^{-3}$ mols per liter) to bring the solution used in these experiments to the isoelectric point, which is,

[12] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.
[13] Loeb, J.,[1] p. 52; *J. Gen. Physiol.*, 1920–21, iii, 547.
[14] Loeb, J.,[1] p. 43; *J. Gen. Physiol.*, 1920–21, iii, 92.

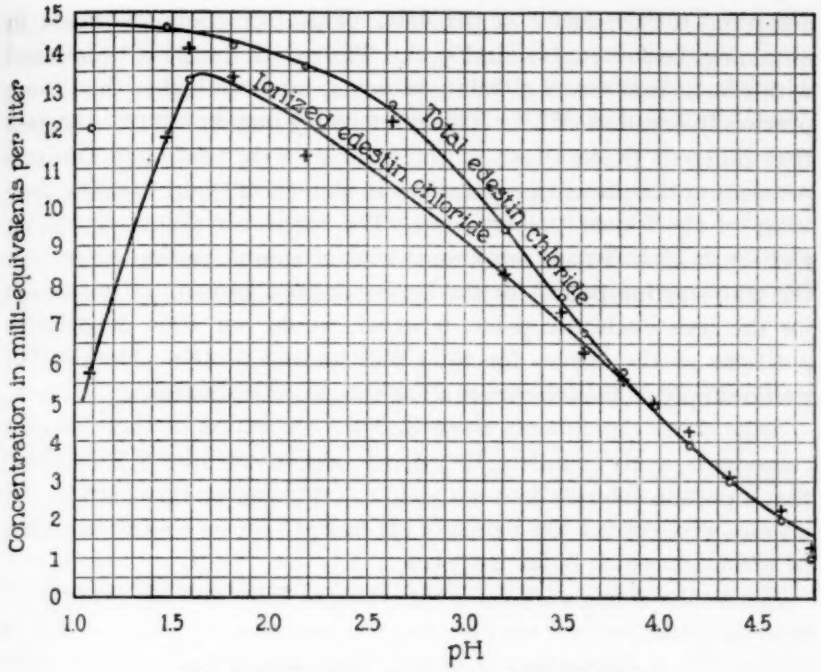


FIG. 4. Ionization of 1 per cent edestin chloride.

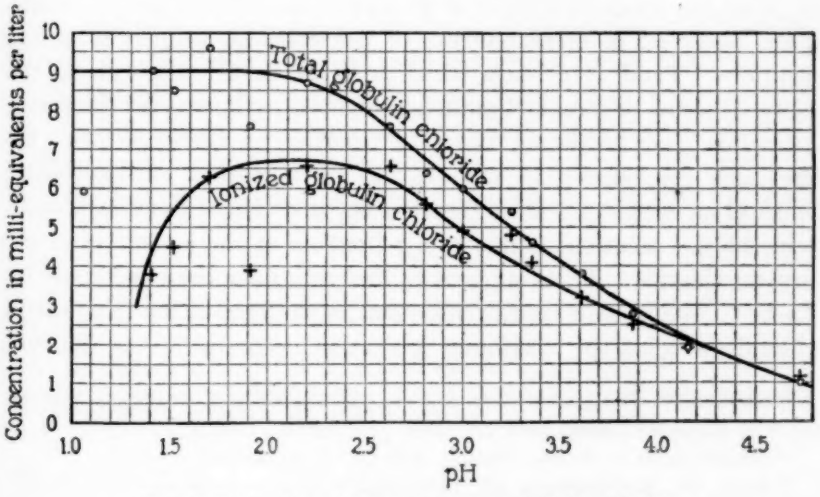


FIG. 5. Ionization of 1 per cent serum globulin chloride.

according to Sörensen,[15] at pH 4.80. This correction was made in obtaining the curves given in Fig. 3. The curves for the $H^+$ combined with edestin and serum globulin, however, are both higher than those previously found at 33°.[16] This discrepancy may be due to the fact that different preparations of the proteins were used, and the suspensions of the present preparations in water were more alkaline than those of the former preparations. The isoelectric points of edestin and serum globulin are not known with certainty, so that at present the true starting points for the curves are not known. A correction for the true isoelectric point, however, would not alter the relative positions of the curves for total and ionized protein chloride, but would simply shift both curves along the axis of ordinates.

Since these experiments show that the ionization of these protein chlorides is nearly complete, they do not support the idea of Robertson[3] that a protein chloride does not yield chloride ions on dissociation. If his idea were correct, the values of pH and pCl for each solution should be identical, which is far from true.

The results of this work agree with those of Pauli and his coworkers[17] in so far as they confirm the idea that the ionization of protein chlorides is like that of ammonium chloride. The present results differ, however, from those quoted by Pauli with regard to any connection between the maximal ionization of the protein chloride and the maximum observed in measurements of the colloidal properties of the solutions. Pauli gives data which indicate that in the case of the dialyzed serum or serum albumin used in his experiments the maximum ionization and maximum viscosity occurred at the same pH. He uses this coincidence as support for his idea that the depressing effect of an excess of acid on the viscosity and other properties of protein chloride solutions is due to a repression of the ionization of the protein chloride. Facts opposed to this view have been presented by Loeb.[18] The present results show that in the case of the five protein chlorides studied the maximum ionization occurs at a very different

[15] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, Copenhagen, 1915–17, xii, 163; *Z. physiol. Chem.*, 1918, ciii, 210.

[16] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597; 1922–23, v, 35.

[17] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920, pt. 1, 45–64.

[18] Loeb, J.,[1] pp. 17–19, 69, 114–119, 130, 197.

pH from the maxima for various colloidal properties. This is brought out clearly by Table III, which is an amplification of the table given by Loeb,[19] including more recent work.[20,16] In so far as the table is complete the figures are quite at variance with Pauli's explanation of colloidal behavior.

The theory of colloidal behavior proposed by Loeb is based on the idea that proteins are amphoteric electrolytes and on Donnan's theory of membrane equilibrium. The former idea suggested that protein chlorides should be highly ionized salts, and this has been confirmed

TABLE III.

*pH Values Corresponding with Maxima in Various Properties of 1 Per cent Protein Chloride Solutions.*

| | Gelatin. | Egg albumin. | Casein. | Edestin. | Serum globulin. |
|---|---|---|---|---|---|
| Osmotic pressure | 3.4 | 3.4 | 3.0 | 3.0 | 3.0 |
| Swelling | 3.2 | — | — | — | — |
| Viscosity | 2.9 | No maximum. | 3.0 | — | — |
| Membrane potential | 4.1 | 3.6 | 3.6 | 4.3 | 3.4 |
| Ionization | 1.4 | 1.6 | 2.2 | 1.6 | 2.2 |

by the present work. The equations for Donnan's theory of membrane equilibrium were deduced on the assumption of complete ionization of the electrolytes present, and the present work lends support to the validity of this assumption with regard to protein chlorides. The present results, therefore, while not in agreement with the other theories mentioned, are in complete agreement with the theory of colloidal behavior which has been applied to the proteins by Loeb.

## IV.

## SUMMARY.

1. By the use of the silver-silver chloride electrode, measurements have been made of the chloride ion concentrations of 1 per cent solutions of five proteins, containing from 0.001 N to 0.1 N hydrochloric

[19] Loeb, J.,[1] p. 115.

[20] Loeb, J.,[1] pp. 122, 199; *J. Gen. Physiol.*, 1921–22, iv, 362; *J. Am. Chem. Soc.*, 1922, xliv, 1930.

acid. The hydrogen ion concentrations of the same solutions have been measured by the use of the hydrogen electrode.

2. The measurements indicate that the chlorides of gelatin, egg albumin, casein, edestin, and serum globulin are highly ionized electrolytes, ionizing to yield chloride ion and a positive protein-hydrogen ion. Their ionization is therefore similar to that of ammonium chloride.

3. The results do not support the idea that a protein chloride does not yield chloride ion on dissociation. They are not in agreement with the idea that the depressing effect of an excess of HCl on the viscosity and other colloidal properties of a protein chloride solution is due to a repression of the ionization of the protein chloride. The results are, however, in complete accord with the theory of colloidal behavior advocated by Loeb.

The writer wishes to express his gratitude to Dr. Jacques Loeb for his supervision of this work.

# THE INFLUENCE OF ELECTROLYTES ON THE CATAPHORETIC CHARGE OF COLLOIDAL PARTICLES AND THE STABILITY OF THEIR SUSPENSIONS.

## II. Experiments with Particles of Gelatin, Casein, and Denatured Egg Albumin.

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(Received for publication, August 22, 1922.)

### I.

Hardy observed that the stability of suspensions of particles of boiled white of egg was a minimum at the isoelectric point and he correlated this observation with another fact discovered by him that the particles are not charged at the isoelectric point, assuming that the stability of suspensions is determined by the electrostatic repulsion of the particles on account of the fact that they are surrounded by double electrical layers. He furthermore explained the precipitation of colloidal suspensions by salts or electrolytes in general by the assumption of an annihilation of the electrical charges of the particles.[1]

Since many proteins are least stable at the isoelectric point, the conclusion was drawn by some authors that even genuine proteins never form crystalloidal solutions, but that their particles are surrounded in water by electrical double layers and are prevented from coalescing through the electrostatic repulsion determined by these layers. The precipitation of genuine proteins from their aqueous solutions by salts was then also explained by the assumption that it was due to the annihilation of these double layers.

While Hardy's observations and conclusions were correct as far as the insoluble particles of boiled white of egg were concerned, it was difficult to harmonize them with observations on certain genuine

[1] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

proteins. Aqueous solutions of crystalline egg albumin at the pH of the isoelectric point will remain stable for a year (if not permanently) if the temperature is not too high, and since the particles have no cataphoretic charge at this pH, it is obvious that they must be kept in solution by forces other than those due to cataphoretic charges, on which the stability of solutions of the same protein depends when it is denatured by heat. The only other forces which can keep proteins in solution are the forces which determine the solubility of crystalloids. This idea is supported in the case of solutions of genuine crystalline egg albumin by viscosity measurements. The writer has shown that whenever a protein solution contains suspended particles (capable of occluding water and of swelling) the viscosity is of a much higher order of magnitude than that of solutions of crystalloids, and that this high viscosity is influenced by acids and alkalies in a similar way as is the osmotic pressure. He found that the viscosity of solutions of crystalline egg albumin is of a comparatively low order of magnitude and is not influenced by acids or alkalies in the same way as is the osmotic pressure.[2] From these observations on the viscosity of the solutions of crystalline egg albumin as well as from the fact that the solutions of this protein of a concentration of 8 per cent or more are stable at the isoelectric point, the inference must be drawn that the forces which keep genuine crystalline egg albumin in solution are of the same nature as the forces which keep crystalloids in solution. These latter forces are, according to Langmuir and Harkins, forces of chemical attraction between solute (or certain chemical groups of the solute) and the solvent—in this case water. These forces may or may not be secondary valency forces. There is a criterion which permits us roughly to decide which of the two kinds of forces determines the stability of solutions, the forces active in crystalloidal solutions (secondary valency forces) or the forces active in colloidal suspensions (electrical double layers). The P.D. of electrical double layers is reduced to zero by comparatively low concentrations of salts and the active ion has always the opposite sign of charge to that of the particle. No such relation exists in the case of crystalloidal solutions, and salts might even increase the solubility of a crystalloid in water.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 195; *J. Gen Physiol.*, 1920–21, iii, 827.

When this criterion is applied to solutions of certain genuine proteins such as crystalline egg albumin or gelatin, it is found that high concentrations of salts, *e.g.* NaCl or $CaCl_2$, are required for precipitation and that the effective ion of the salt has no relation to the sign of charge of the protein particle. Thus sulfates are generally better precipitants for solutions of genuine egg albumin and gelatin than chlorides, regardless of whether the protein is negatively or positively charged, or not charged at all. To escape the conclusion that genuine proteins form true solutions in water, some colloid chemists suggested that genuine proteins should be called emulsoids, thereby insinuating that such proteins as genuine egg albumin or gelatin form diphasic systems of the type of emulsions of oil in water. It is true that the stability of droplets of pure oil in water is also due to the existence of electrical double layers surrounding the particles, but unfortunately for the colloidal speculations on "emulsoids" Powis has shown that the P.D. surrounding droplets of oil is destroyed by the same low concentration of salts as the P.D. of "suspensoids," and that in the case of emulsions of oil in water the effective ion of the salt has the opposite sign of charge from that of the oil drop.[3]

All amino-acids form crystalloidal solutions but some, like alanine, are very soluble while others, like tyrosine, are sparingly soluble in water. Similar differences must exist between proteins, according to the nature and linkage of the amino-acids of which they are composed. When the forces of attraction between the molecules of the solute and the solvent become smaller than the forces of attraction between the molecules of the solute (or of certain groups like hydrocarbons) for each other, aggregates of molecules will be formed. If it happens that electrical double layers are formed around each nascent aggregate, no flocculation need occur, the aggregates forming stable suspensions. Flocculation of such suspensions can be brought about when the P.D. of the double layer surrounding each particle is lowered below a critical limit by the addition of electrolytes. It is, however, quite possible that the addition of salt may increase the forces of attraction between the solute and water. It may also happen, as was demonstrated by Northrop and De Kruif, that a salt may diminish the forces of cohesion between the micellæ and in that case

[3] Powis, F., *Z. physik. Chem.*, 1914–15, lxxxix, 186.

no flocculation of aggregates will occur even if the salt annihilates the P.D. between particle and water.[4] In view of all these possibilities it is necessary actually to measure the P.D. between protein particles and water before any decision can be reached whether or not proteins are kept in solutions by electrical double layers or by those forces which determine the solution of crystalloids. The writer has measured the influence of electrolytes on the P.D. of the electrical double layer surrounding protein particles to separate the influence of these forces from the influence of forces which determine the stability of crystalloidal solutions and it is the intention of this paper to give the results of these measurements. The question concerning the origin of these electrical double layers will be discussed in a subsequent paper.

II.

Three sets of experiments were made: namely, first, with collodion particles coated with gelatin; second, with casein particles containing no collodion; and third, with particles of denatured egg albumin.

For the first set of experiments, with collodion particles coated with gelatin, the same collodion particles were used as in the preceding paper.[5] To a thick suspension of these particles was added enough isoelectric gelatin to make a 1 per cent gelatin solution. The water used for the solution had a pH of 4.7; *i.e.*, the pH of the isoelectric point of gelatin. The collodion particles remained in the solution over night. The next day the suspension in gelatin was heated to about 40°C. to make sure that the gelatin was completely liquefied, and the collodion particles were centrifuged out from the gelatin solution while the latter was still warm. The thick sediment of collodion particles which was centrifuged out was suspended in 50 cc. of water of pH 4.7 and kept as a stock suspension. This stock suspension was too concentrated for microscopic measurement of the migration in an electric field and a few drops of the stock suspension were usually put into 50 cc. of a solution of an electrolyte, shaken up, and left standing for 30 minutes at about 24°C. This suspension was then used for microscopic measurement of the velocity in an electric field.

[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655.
[5] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

In the first sets of experiments the gelatin-collodion particles were suspended in various concentrations of HCl, $H_2SO_4$, NaOH, and $Ba(OH)_2$, and Fig. 1 gives the cataphoretic P.D. as calculated from the mobility measurements. The abscissæ are the equivalent concentrations of acid and alkali. On account of the Donnan equilibrium the pH of the surface film of gelatin on the collodion particles was higher than that of the acid solution used. The ordinates are

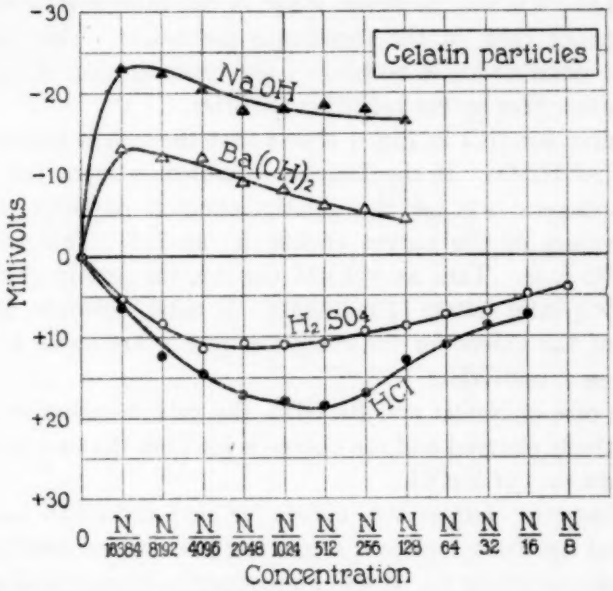


FIG. 1. Influence of acids and alkalies on the cataphoretic P.D. of collodion particles coated with isoelectric gelatin. Abscissæ are the normality of acids or alkalies in solution; ordinates are the cataphoretic P. D. of the particles in millivolts. The ordinates are above the zero line when the particles are negatively charged, and below when they are positively charged.

the P.D. in millivolts; when the particles were negatively charged the ordinates were plotted above the zero line, so as to make the curves comparable to the curves for the P.D. of the collodion particles free from protein published in the preceding paper. When the collodion-gelatin particles were positively charged, the ordinates were plotted below the zero line.

Fig. 1 shows that at the isoelectric point the P.D. was zero, that in

acid the particles were positively charged, and in alkali negatively charged. This was to be expected. The new part in our experiments is the variation of the P.D. with the concentration of acid or alkali used. In $H_2SO_4$ the P.D. curve reaches a maximum at a concentration of about N/4,096 and then falls slowly. This agrees approximately with the membrane potentials between $H_2SO_4$ and gelatin sulfate solutions, as shown on page 129 of the writer's book. The absolute values of the P.D. are, however, lower in the cataphoretic P.D. curve in this paper than in the membrane potentials. This cannot be explained since we know nothing of the concentration of the gelatin in the surface film on the collodion particles.

The curve for HCl in Fig. 1 shows that the P.D. is higher than in the case of $H_2SO_4$. If the Donnan equilibrium were the cause of the cataphoretic P.D. of the gelatin-collodion particles, the ratio of the values of the curves should be as 2:3. This is approximately the case. Thus at N/1,024 the P.D. for gelatin chloride was 18 and for gelatin sulfate 11 millivolts. It must, however, be remembered that the values for the cataphoretic P.D. are liable to have an error of $\pm$ 2 millivolts.

In the case of NaOH and $Ba(OH)_2$ the gelatin-collodion particles are negatively charged and the charge is lower in the case of $Ba(OH)_2$ than in the case of NaOH.

The character of these P.D. curves for acid and alkali has a great theoretical significance since it seems to eliminate one possible source for the cataphoretic P.D. It had been suggested that colloidal micellæ migrate in the galvanic field not on account of an electrical double layer surrounding them but on account of the drag on the colloidal ions contained in the micella. If this were the case, the P.D. curves for NaOH or $Ba(OH)_2$ should be interpreted to mean that in a concentration of M/16,000 NaOH or $Ba(OH)_2$ the ionization of Na gelatinate was a maximum and that its ionization was repressed at higher concentrations of these alkalies, and that the ionization of gelatin chloride was a maximum in M/512 HCl and was repressed at higher acid concentrations. Both assumptions would be contrary to fact.[6] This leaves only one possible explanation for the migration of the gelatin

[6] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 116–119.

micellæ in an electric field; namely, that it must be due to the formation of an electrical double layer between particles and surrounding aqueous solution.

The stock suspension of gelatin-coated particles had a pH of 4.7, and several drops of this suspension were put into 50 cc. of different concentrations of a solution of electrolytes, NaCl, $Na_2SO_4$, $Na_4Fe(CN)_6$, $CaCl_2$, and $LaCl_3$, all of a pH of 4.7, and the sign and rate of migration were observed. In this case it is very essential that the pH of the solution should be exactly 4.7, since a slight deviation from this pH will cause a migration of the particles. Without the addition of salt, the P.D. of the particles was found in the five series to be −1.5, −1.7, +1.0, −3.0, −1.8 millivolts respectively; in other

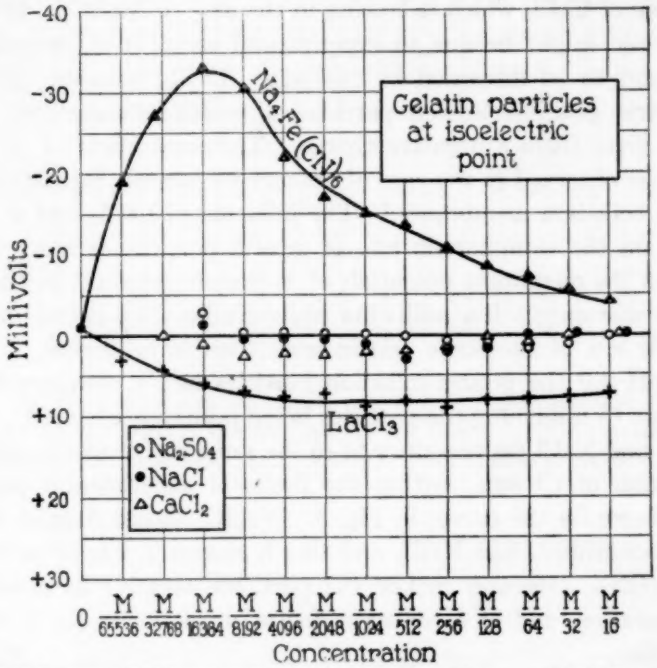


FIG. 2. Influence of different salts on the cataphoretic P. D. of gelatin-collodion particles at pH 4.7 (isoelectric point). Without salts the particles are not charged. In $Na_4Fe(CN)_6$ the particles are negatively charged; in $LaCl_3$ they are positively charged. The effect of NaCl, $Na_2SO_4$, and $CaCl_2$ is scarcely noticeable, except that in $CaCl_2$ the particles are slightly more positive than in either NaCl or $Na_2SO_4$.

words, there was practically no migration of the particles at the isoelectric point. This agrees with the results of the previous observers.

In our experiments on anomalous osmosis the conclusion was reached that the presence of NaCl, $Na_2SO_4$, and $CaCl_2$ leaves isoelectric gelatin particles uncharged. Fig. 2 shows that the gelatin-coated collodion particles have possibly a very minute positive charge in the presence of $CaCl_2$ which is lacking in the case of $Na_2SO_4$. It is very small, and might be due to experimental error; it is, however, too persistent to be discarded in this way. $LaCl_3$, however, gives the isoelectric gelatin-collodion particles a positive charge and $Na_4Fe(CN)_6$ gives them a negative charge. The same effect of these two salts was observed in the case of anomalous osmosis through gelatin-coated collodion membranes.[7] The influence of $LaCl_3$ and of $Na_4Fe(CN)_6$ on the cataphoretic P.D. of gelatin particles is much greater than on the membrane potentials of isoelectric gelatin. In the latter case it was only a few millivolts while $Na_4Fe(CN)_6$ raised the cataphoretic P.D. of isoelectric gelatin more than 30 millivolts.

At pH 4.0 the gelatin-collodion particles had a cataphoretic P.D. of about 13 millivolts, the particles being positively charged. $LaCl_3$, $CaCl_2$, and NaCl depress the P.D. to the same extent at the same concentration of Cl ions (within the limits of experimental accuracy) as is shown by the curves in Fig. 3. $Na_2SO_4$ should depress the P.D. more powerfully than NaCl, and this is shown in Fig. 3 to be true. $Na_4Fe(CN)_6$, however, makes the particles negative at a very low concentration, and at a concentration of M/2,048 the P.D. is over 20 millivolts.

Fig. 4 gives the influence of the four salts NaCl, $CaCl_2$, $LaCl_3$, and $Na_2SO_4$ on the P.D. of gelatin-collodion particles at pH 3.0. The effect of the first three salts is about the same while $Na_2SO_4$ has a much stronger depressing effect.

At pH 5.8 the cataphoretic P.D. was about 14 millivolts without the addition of salts, the gelatin particles being negatively charged (Fig. 5). In this case they should be influenced by salts in the same way as the collodion particles not treated with gelatin, since such collodion particles are also negatively charged. While, however, NaCl, $Na_2SO_4$, and $Na_4Fe(CN)_6$ raise the P.D. of collodion particles not

[7] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

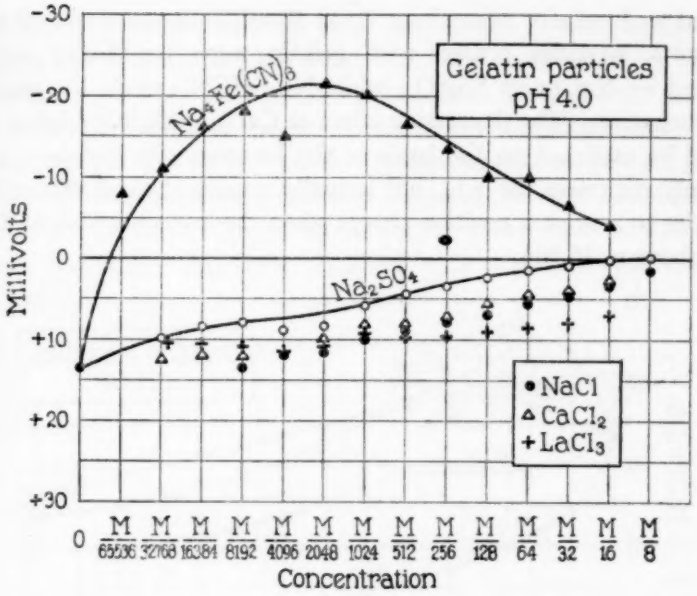


FIG. 3. Influence of salts on the cataphoretic P. D. of gelatin-collodion particles at pH 4.0. In $Na_4Fe(CN)_6$ the particles are negatively, in the other salts positively charged. In $Na_2SO_4$ the particles are charged less than in NaCl, $CaCl_2$, or $LaCl_3$.

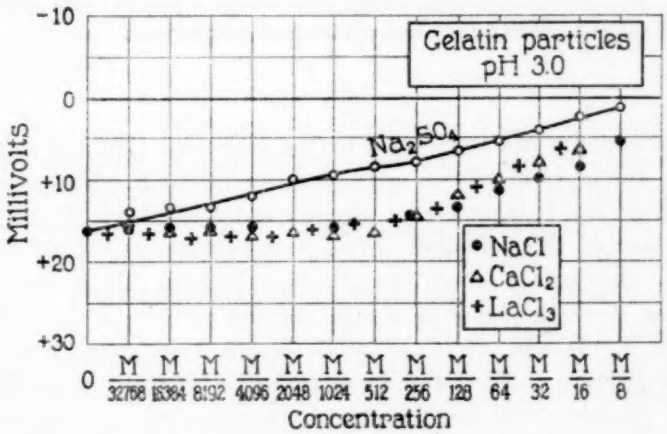


FIG. 4. Influence of salts on cataphoretic charges of gelatin-collodion particles at pH 3.0. The particles are positively charged.

treated with gelatin from about 20 to 30 millivolts to about 60 to 70 millivolts, particles coated with gelatin were practically not influenced by NaCl and $Na_2SO_4$, while $Na_4Fe(CN)_6$ made the particles more negative. The depressing effect of $CaCl_2$ is slightly higher than should be expected on the basis of the Donnan effect alone. $LaCl_3$ not only depresses the P.D., but actually causes the collodion-gelatin particles to assume a positive charge when the concentration of $LaCl_3$ rises above M/16,384.

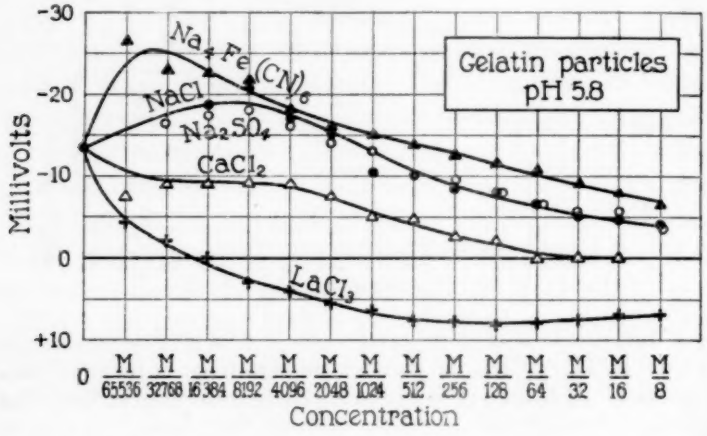


FIG. 5. Influence of salts on the cataphoretic charge of gelatin-collodion particles at pH 5.8. $LaCl_3$ reverses the negative charge of the particles.

III.

These experiments were open to the objection that the collodion beneath the layer of gelatin might influence the P.D. This objection is justified since it is uncertain whether or not the layer of gelatin on the surface of the collodion particles is continuous—there may be spots on the surface where the gelatin is lacking. It is also uncertain how thick the layer of gelatin on the surface of the collodion particles is. It was, therefore, advisable to make experiments with another genuine protein, which can easily be obtained in the form of minute granules, without the aid of some foreign material such as collodion. Such material was available in the form of isoelectric casein, which if properly prepared can be obtained in very minute

solid particles, which dissolve only very slowly in acid solution. In alkaline solution, *i.e.* at a pH above 7.0, they dissolve rapidly but it is not necessary for our purpose to experiment at a pH above 5.8. Since the isoelectric point of casein is practically identical with that of gelatin, *i.e.* pH 4.7, all the mobility measurements made with gelatin-coated collodion particles could thus be repeated with casein particles free from collodion. The results obtained with casein particles were almost identical with those obtained with gelatin-coated collodion particles.

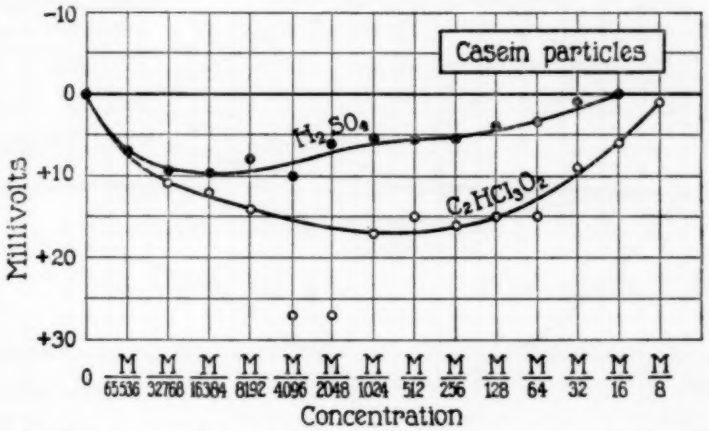


FIG. 6. Influence of trichloroacetic and sulfuric acids on the cataphoretic P.D. of casein particles.

The method of evaluating the cataphoretic P.D. of the casein particles was the same as that used for evaluating the cataphoretic P.D. of the gelatin-coated particles. Fig. 6 gives the effects of different concentrations of trichloroacetic and sulfuric acids on the cataphoretic P.D. of originally isoelectric particles of casein. Trichloroacetic acid was selected because casein is practically as insoluble in this acid as in sulfuric acid. At pH 4.7 the P.D. of the casein particles was zero. It increased with increasing concentrations of acid until it reached a maximum at a molecular concentration of between M/4,096 and M/256, and then diminished again with increasing concentration of acid. The particles were positively charged.

Fig. 7 shows the influence of five salts, $LaCl_3$, $CaCl_2$, NaCl, $Na_2SO_4$,

and $Na_4Fe(CN)_6$ on the cataphoretic P.D. of the casein particles at the isoelectric point; *i.e.*, pH 4.7. As was to be expected the $LaCl_3$ causes the isoelectric casein particles to be positively charged, and $Na_4Fe(CN)_6$ causes them to be negatively charged. Fig. 7 shows also that the particles are slightly more negative in the presence of $Na_2SO_4$ than in the case of NaCl, and more so in this case than in that

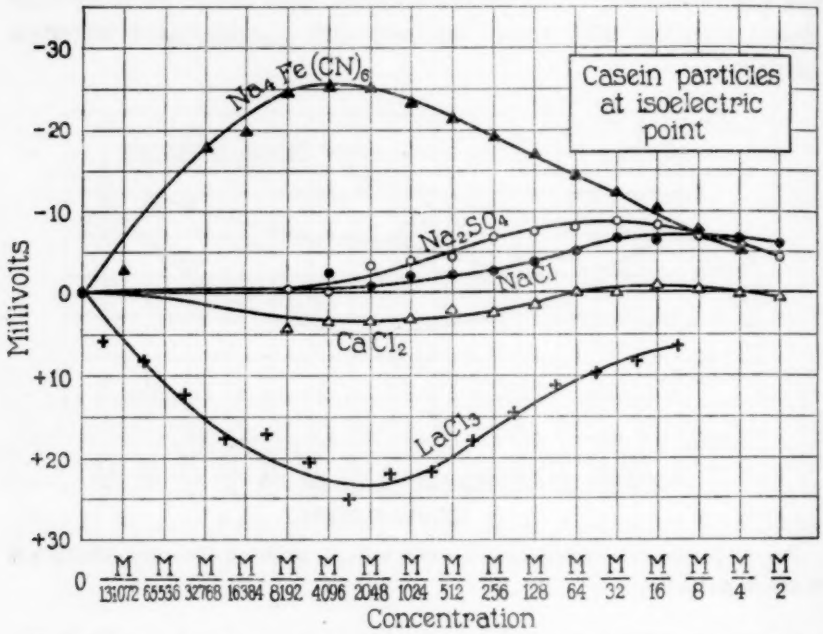


FIG. 7. Influence of salts on the cataphoretic P. D. of casein particles at pH 4.7 (isoelectric point). Without salts the particles are not charged. In the presence of $Na_4Fe(CN)_6$ they are negatively, and in $LaCl_3$ they are positively charged. In $Na_2SO_4$ the particles are slightly negatively, in $CaCl_2$ slightly positively charged.

of $CaCl_2$. The latter salt may make the casein particles even slightly positive. The effects of these three salts, though slight, cannot be ascribed to an error.

The next group of measurements was made at pH 4.0 (Fig. 8). The acid used to bring the particles to this pH was HCl and hence the casein salt formed was casein chloride. The cataphoretic P.D. of the casein particles was about 14 millivolts, in the absence of salts,

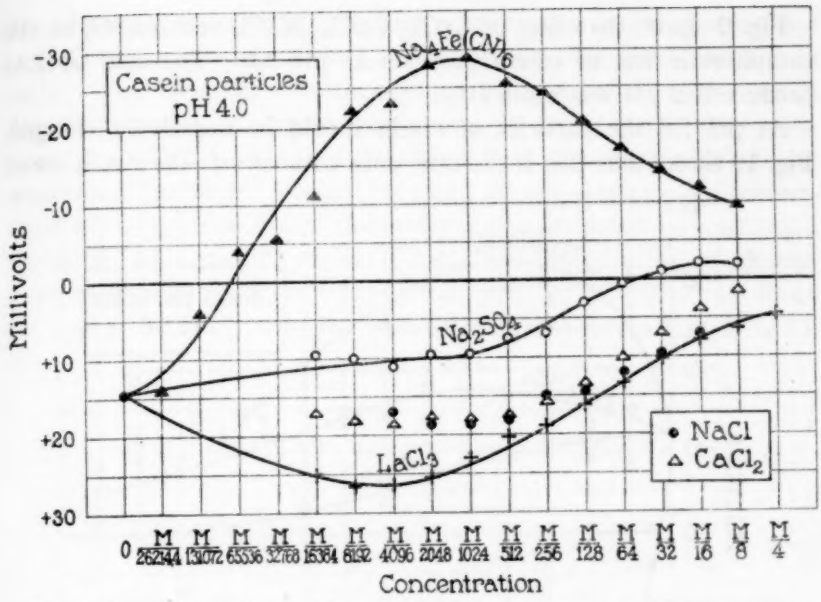


FIG. 8. Influence of salts on the cataphoretic P. D. of casein particles at pH 4.0.

the particles being positively charged. $Na_2SO_4$ diminished the P.D. more than NaCl, $CaCl_2$, or $LaCl_3$. $Na_4Fe(CN)_6$ reverses the sign of charge of the protein particles in concentrations above M/65,000.

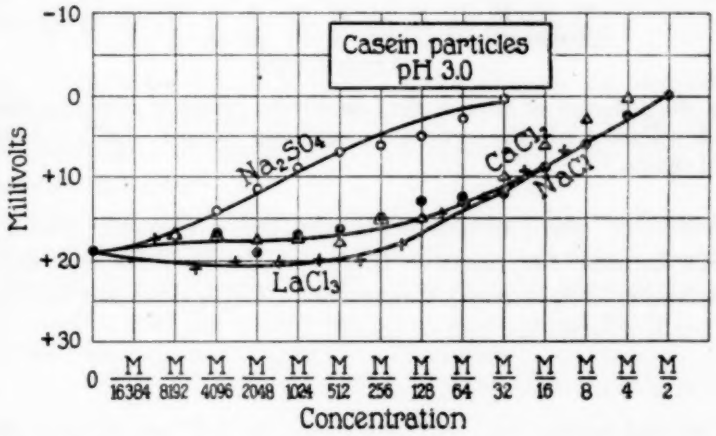


FIG. 9. Influence of salts on the cataphoretic P. D. of casein particles at pH 3.0.

Fig. 9 shows the effect of $LaCl_3$, $CaCl_2$, NaCl, and $Na_2SO_4$ on the cataphoretic P.D. of casein particles at pH 3.0. The acid used to produce this pH was again HCl.

At pH 5.8 the particles of casein should be negatively charged. Fig. 10 shows that this is the case since without salt the P.D. is about

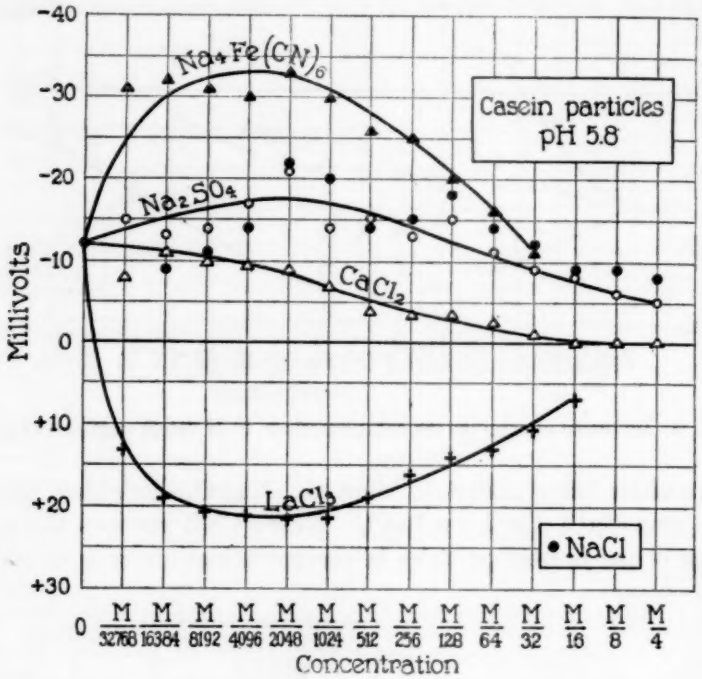


FIG. 10. Influence of salts on the cataphoretic charge of casein particles at pH 5.8. Without salts the particles are negatively charged. $LaCl_3$ reverses the sign of charge.

12 millivolts, the particles being negatively charged. NaCl and $Na_2SO_4$ increase the negative charge of the particles but slightly, while $Na_4Fe(CN)_6$ increases it markedly. $CaCl_2$ depresses the charge and $LaCl_3$ reverses the sign of charge.

IV.

It seemed advisable to investigate also the influence of salts on the cataphoretic P.D. of denatured crystalline egg albumin. Denatured

egg albumin was prepared by heating a 1 per cent solution of crystalline egg albumin of pH 4.8 to 90°C. allowing the coagulated particles to settle. The sediment was then ground up in a mortar with a small amount of water to a milky suspension. 4 drops of this suspension were then added to 50 cc. of the various solutions used. This latter suspension was always allowed to stand 20 minutes at room temperature in the solution before the mobility measurements in Northrop's apparatus were made. It was found that the influence of electrolytes on the cataphoretic P.D. of denatured egg albumin was about the same as on casein and gelatin particles, as is shown in Figs. 11 to 15.

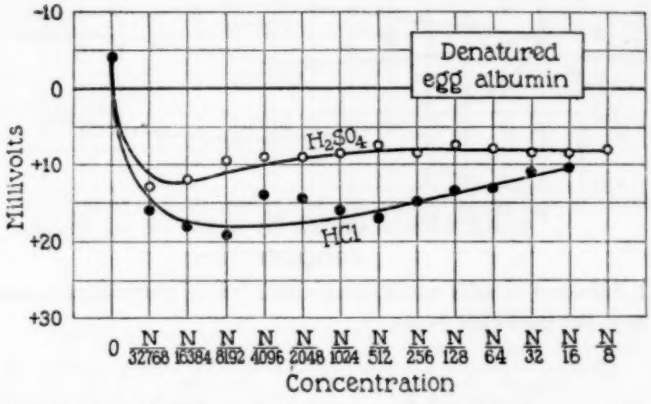


FIG. 11. Influence of HCl and $H_2SO_4$ on the cataphoretic P.D. of particles of denatured egg albumin.

Fig. 11 gives the influence of HCl and $H_2SO_4$ on the cataphoretic P.D. of particles of denatured egg albumin. The influence of the two acids corresponds almost to that to be expected on the basis of the Donnan equilibrium.

Fig. 12 shows the influence of the five salts, NaCl, $Na_2SO_4$, $CaCl_2$, $LaCl_3$, and $Na_4Fe(CN)_6$ on the cataphoretic P.D. of almost isoelectric egg albumin (pH 5.0 instead of 4.8). The particles are uncharged and NaCl, $Na_2SO_4$, and $CaCl_2$ have almost no influence on the P.D., while $LaCl_3$ acts like acid and $Na_4Fe(CN)_6$ like alkali.

At pH 4.0 (Fig. 13) the particles of egg albumin have a positive charge and the P.D. is about 20 millivolts. $Na_4Fe(CN)_6$ reverses the charge at a concentration of M/16,000, while $LaCl_3$ increases the

charge slightly. NaCl and $CaCl_2$ have only a depressing effect due to the Cl ions, and $Na_2SO_4$ has a much stronger depressing effect. $Na_2SO_4$ does not reverse the sign of charge as does $Na_4Fe(CN)_6$.

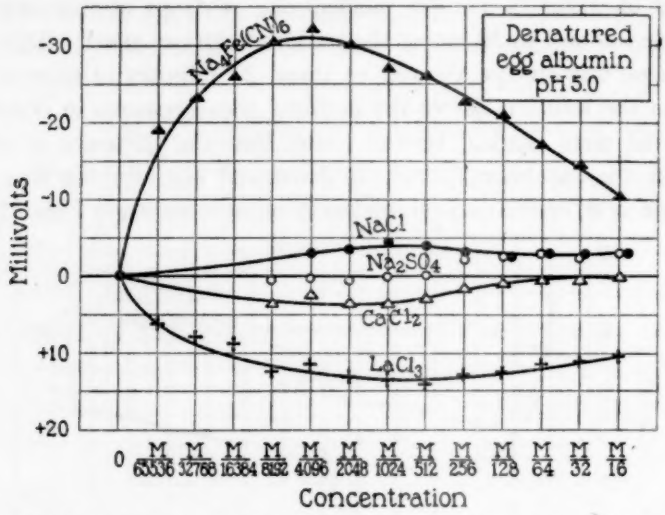


FIG. 12. Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin near the isoelectric point.

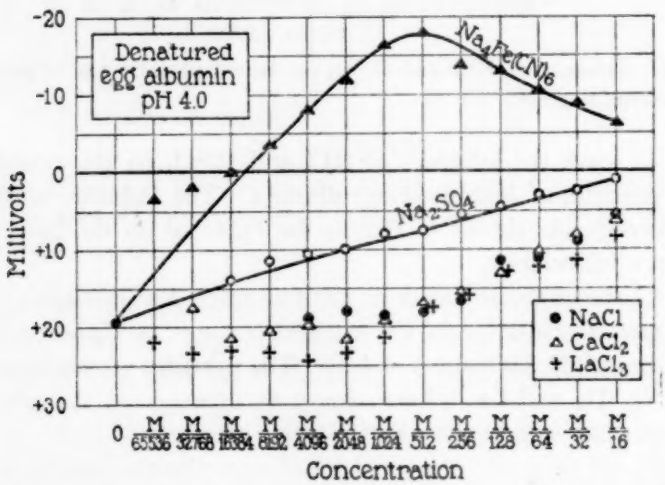


FIG. 13. Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 4.0.

Fig. 14 shows the effects of NaCl, $CaCl_2$, $LaCl_3$, and $Na_2SO_4$ on the P.D. of egg albumin at pH 3.0, and Fig. 15 shows the effects of salts on the P.D. of Na albuminate at pH 5.8. In this latter case the protein

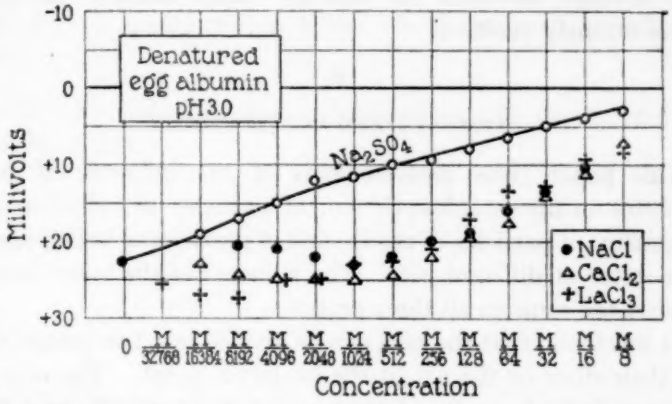


FIG. 14. Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 3.0.

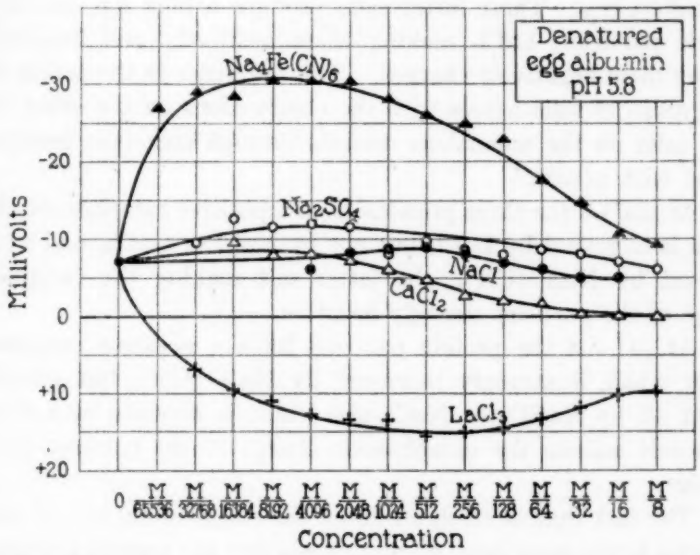


FIG. 15. Influence of salts on the P.D. of particles of denatured egg albumin at pH 5.8.

has a negative charge and the P.D. was about 8 millivolts. NaCl, $Na_2SO_4$, and $CaCl_2$ had practically only a depressing effect, which was greater in the case of $CaCl_2$ than in the case of NaCl or $Na_2SO_4$. $LaCl_3$, however, reversed the sign of charge making the protein particles strongly positive.

## V.

## SUMMARY AND CONCLUSIONS.

1. This paper gives measurements of the influence of various electrolytes on the cataphoretic P.D. of particles of collodion coated with gelatin, of particles of casein, and of particles of boiled egg albumin in water at different pH. The influence of the same electrolyte was about the same in all three proteins.

2. It was found that the salts can be divided into two groups according to their effect on the P.D. at the isoelectric point. The salts of the first group including salts of the type of NaCl, $CaCl_2$, and $Na_2SO_4$ affect the P.D. of proteins at the isoelectric point but little; the second group includes salts with a trivalent or tetravalent ion such as $LaCl_3$ or $Na_4Fe(CN)_6$. These latter salts produce a high P.D. on the isoelectric particles, $LaCl_3$ making them positively and $Na_4Fe(CN)_6$ making them negatively charged. This difference in the action of the two groups of salts agrees with the observations on the effect of the same salts on the anomalous osmosis through collodion membranes coated with gelatin.[7]

3. At pH 4.0 the three proteins have a positive cataphoretic charge which is increased by $LaCl_3$ but not by NaCl or $CaCl_2$, and which is reversed by $Na_4Fe(CN)_6$, the latter salt making the cataphoretic charge of the particles strongly negative.

4. At pH 5.8 the protein particles have a negative cataphoretic charge which is strongly increased by $Na_4Fe(CN)_6$ but practically not at all by $Na_2SO_4$ or NaCl, and which is reversed by $LaCl_3$, the latter salt making the cataphoretic charge of the particles strongly positive.

5. The fact that electrolytes affect the cataphoretic P.D. of protein particles in the same way, no matter whether the protein is denatured egg albumin or a genuine protein like gelatin, furnishes proof that the solutions of genuine proteins such as crystalline egg albumin or gelatin

are not diaphasic systems, since we shall show in a subsequent paper that proteins insoluble in water, *e.g.* denatured egg albumin, are precipitated when the cataphoretic P.D. falls below a certain critical value, while water-soluble proteins, *e.g.* genuine crystalline egg albumin or gelatin, stay in solution even if the P.D. of the particles falls below the critical P.D.

The mobility measurements mentioned in this paper were made by Mr. M. Kunitz.

# THE MECHANISM OF THE EFFECT OF ACIDS AND ALKALIES ON THE DIGESTION OF PROTEINS BY PEPSIN OR TRYPSIN.

## A Correction.

By JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 27, 1922.)

In a paper of the above title[1] the following statement was made: ". . . . the amount of acid required to bring the protein solution to a given pH is independent of the nature and valence of the anion." This statement is literally true only in the case of strong acids which the writer had in mind at the time. In the case of weak or polyvalent acids the amount required is larger the weaker the acid. This point is thoroughly discussed by Loeb.[2]

[1] Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 269.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, Chapter IV, pp. 40 ff.



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